

Methods and compositions to generate and control the effector profile of T cells by simultaneous loading and activation of selected subsets of antigen presenting cells

5 Related Cases:

The present application claims priority to U.S. patent application Serial Number 60/412,219 filed September 20, 2002 and international application number PCT/US 03/07995 filed on March 14, 2003, both of which are hereby incorporated by reference in
10 their entireties.

Field of the Invention

The present invention is generally directed to methods and compositions to generate an immune response. More specifically, the present invention is directed to
15 methods and compositions of loading an antigen-presenting cell to display a delivered epitope on a MHC class I molecule in a context appropriate for the generation of desired T cell responses.

Background of the Invention

20 No direct evidence has been shown that delivery of antigen via Fc gamma receptors ("FcγR") triggers an effective antitumoral or antiinfectious response. For example, it was previously shown that delivery of a viral NP (nucleoprotein) derived epitope within an immunoglobulin or IgG backbone did not result in detectable induction of cytotoxic immunity (Zaghouani et al., Eur J Immunol. 1993 Nov; 23(11):2746-50). In
25 contrast, delivery of the same epitope in context of NP expressing cells (transfectomas) resulted in significant cytolytic activity. It was therefore concluded at that time that "APC (antigen presenting cells) are unable to present an influenza nucleoprotein [NP] peptide from the same context (1 microM Ig-NP) to an MHC class I-restricted T cell " and thus, "the endocytic compartment, when offered MHC class I- and II-restricted peptides within
30 the same carrier protein context, favors presentation by class II by at least 1000-fold".

Access of the NP epitope to MHC class I presentation pathway is dependent on delivery strategy and was thus believed to be severely limited subsequent to FcγR internalization. More recently, it has been proposed that cross-linking or simultaneous

engagement of FcγR on antigen presenting cells ("APC") may greatly optimize signal transduction and result in stimulation of cross-priming and APC stimulation, resulting in effective loading of MHC class I molecules (Regnault et al., J Exp Med. 1999, Jan 18;189(2):371-80). This could be achieved using immune complexes (multivalent antigen-antibody non-covalent complexes); however, due to the potential of C ("complement") mediated disease, the complexes could only be administered to the APC *ex vivo* (Naama et al., J Clin Lab Immunol. 1985 Jun;17(2):59-67; Rafiq et al., J Clin Invest. 2002 Jul;110(1):71-9). Alternatively, (Fab)2-antigen recombinant fusion constructs directed to receptors onto APC, can result in receptor cross-linking internalization, and presentation in context of MHC class II molecules (Lunde et al., Biochem Soc Trans 2002;30(4):500-6). The insertion of antigen, however, modifies the Fc portion of the constant domains (CH2 and CH3) of the immunoglobulin ("Ig") that can result in serious and unpredictable effects on the half life and pharmacokinetics, two parameters that are tightly associated with the integrity of this segment (Spiegelberg HL, J Clin Invest 1975 Sep;56(3):588-94). Finally, there is no conclusive evidence to date that either one of the strategies described above, when applied *in vivo*, induce protective or therapeutic anti-tumoral or anti-microbial immunity that would be associated with the generation of optimal MHC class I and II-restricted T cells that produce specific cytokines such as IFN-γ. Even when applied *ex vivo*, the immune complex strategy has displayed limited efficacy due to the balance in the activity of ITAM+ and ITIM+ FcγR (Kalergis and Ravetch, J Exp Med 2002 Jun 17;195(12):1653-9). Thus, it has yet to be determined whether *in vivo* delivery of antigen to APC via the monovalent ligation of Fcγ receptors can be used to induce effective anti-tumoral or antiviral immunity.

PCT Application Serial Number PCT/US03/07995 filed March 14, 2002 and U.S. patent application serial number 60/364,490 filed April 30, 2002 are hereby incorporated by reference. Swiss-Protein/Trembl Protein Knowledgebase™ on CD-ROM, available from Geneva BioInformatics, is hereby incorporated by reference in its entirety.

Summary of the Invention

The present invention demonstrates, contrary to expectations, that *in vivo* and *ex vivo* loading of APC via monovalent engagement of FcγR, using peptide epitopes

covalently attached to the IgG backbone without modification of the Fc portion, results in access of the epitope to the MHC I processing and presentation pathway, with effective loading of MHC class I molecules. Unexpectedly, this results in generation of robust Tc2 responses characterized by IL-4, but not IL-2 or IFN- γ -producing, MHC class I restricted
5 T cells that recognize the epitope within IgG backbone.

In addition, the generation of this "deviated" response was not effective in controlling a pathologic process associated with tumor growth, nor was it associated with significant priming of cytolytic T cells. This explains largely the previous failure to detect induction of immunity in similar context previously and demonstrates, unexpectedly, that
10 cross-linking or multivalent engagement of Fc γ R on APC (such as in context of immune complexes or Fab2-antigen compounds) is not a prerequisite for effective loading of the peptide onto MHC class I molecules. This is important since the concept could be applied *in vivo* (in contrast to immune complexes) and the integrity of Fc portion and thus PK profile could be retained (in contrast to Fab2-antigen recombinant molecules). Despite
15 effective loading of MHC class I molecules, the APC were not able to trigger protective anti-tumoral and anti-microbial immunity when loaded *in vivo* by peptide epitope within IgG backbone.

Further, the present application discloses novel compositions that result in effective redirection of class I-immunity to Tc1 effectors that take advantage of the
20 unexpected loading of MHC I by peptide within IgG backbone. Such compositions are able to transform seemingly ineffective MHC class II and class I-restricted peptides into highly effective ones. Fc γ R-mediated loading of APC associated with stimulation of APC by novel synthetic polynucleotides, result in generation of class I-restricted cytolytic cells and IFN- γ , IL-2 producing T cells, further associated with protection against a highly
25 virulent microbe or recovery from malignant tumoral process. It is also shown that variants of the technology, applied incorrectly or as previously proposed, are not optimal in generation of immunity protective against viruses or tumors, in particular of MHC class I-restricted nature. The present application demonstrates the reason for past failures and teaches how to obtain and apply the different components of the technology in order
30 to obtain optimal effect.

Various embodiments of the invention include:

1. A method of loading an antigen presenting cell and generating a T cell response against an antigen or peptide epitope by use of at least one peptide epitope attached to an Ig, Ig backbone backbone or portion thereof thereby forming an Ig-peptide molecule/complex or portion thereof wherein when administered to a patient *in vivo* or *ex vivo*, the epitope is effectively processed and presented by the MHC I pathway of the antigen presenting cell resulting in effective loading of MHC class I molecules on the antigen presenting cell thereby resulting in an MHC class I – peptide complex.
2. The method of paragraph 1 wherein the Ig-peptide molecule/complex or portion thereof is administered with RNA strands.
3. The method of paragraph 2 wherein the RNA is dsRNA strand and is pA:pU.
4. The method of paragraph 3 wherein the dsRNA is pA:pU and the dsRNA is between approximately 20 - 100 base pairs in size.
5. The method of paragraphs 1, 2, 3 or 4 wherein the Ig backbone is derived from human Ig.
6. The method of paragraphs 1, 2, 3 or 4 wherein the Ig backbone is derived from human IgG.
7. The method of paragraph 1, 2, 3, or 4 wherein the Ig backbone is humanized Ig.
8. The method of paragraph 1 wherein the antigen presenting cell is loaded via monovalent engagement of FcγR.
9. The method of paragraph 1 wherein the antigen presenting cell may be loaded *in vivo* or *ex vivo*.

10. The method of paragraph 1 wherein the peptide epitopes are covalently attached to the Ig backbone.

5 11. The method of paragraph 1 wherein the peptide epitope is attached to the Ig backbone without modification of the Fc portion of the Ig.

12. The method of paragraph 1 wherein the peptide epitope is inserted within a CDR region of the immunoglobulin molecule.

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13. The method of paragraphs 1, 2, 3 or 4 wherein the peptide epitope is inserted within a CDR region of the immunoglobulin molecule by insertion or deletion.

14. The method of paragraphs 1, 2, 3 or 4 wherein the MHC class I-peptide complex
15 results in generation of robust Tc2 responses characterized by IL-4 but not IL-2 or IFN- γ -production.

15. The method of paragraph 1 wherein the peptide epitope is selected from the group consisting of: influenza virus M1 or M2; hepatitis C virus NS3; hepatitis B virus core
20 antigen; human papilloma virus HPV 18-E7, HPV 16 – E7, HPV 18 E6, HPV 16 E6; melanoma –gp100; MART-1; TRP-2; carcinoembryonic antigen precursor; Her –2; tetanus toxin universal T helper epitope; HIV-1: reverse transcriptase; HIV1: gag; insulin precursor – human; human Gad 65; prostate tumor antigens; mucin 1; herpes simplex antigens; and, respiratory syncytial virus antigens.

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16. The method of paragraph 1 wherein the negative effects of sera are avoided.

17. The method of paragraphs 1, 2, 3 or 4 wherein the Ig peptide molecule and dsRNA are administered by subcutaneous or intraperitoneal injection.

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18. The method of paragraph 1 wherein the antigen presenting cell is selected from the group consisting of dendritic cells, monocytes, macrophages and B cells.
19. The method of paragraph 1 wherein the antigen presenting cell is selected from the group consisting of CD11c+ and CD11b+ APC.
20. The method of paragraph 1 wherein the resulting MHC-peptide complexes formed by *in vivo* delivery are expressed for up to 1 to 2 weeks.
21. The method of paragraphs 1, 2, 3 or 4 wherein the MHC-peptide complex results in activation of T cells.
22. The method of paragraph 21 wherein the T cell response is determined by ITAM+ and ITIM+ Fcγ receptors on APC.
23. The method of paragraph 21 wherein expression of the gamma chain of ITAM+ FcγR isoforms induces the T cell response wherein ITIM+ FcγRII limits the T cell response.
24. The method of paragraphs 18 or 19 wherein monocytes induce Th2 and Tr1 cells, both dendritic cells and monocytes induce Th3 cells, and wherein CD11b+ monocytes are more potent than dendritic cells in triggering a regulatory response following IgG-mediated delivery of T cell epitope.
25. The method of paragraphs 1, 2, 3 or 4 wherein the loading of APC with a peptide delivered within an Ig backbone *in vivo* results in induction of Th2 immunity.
26. The method of paragraphs 1, 2, 3 or 4 wherein the loading of APC with a peptide delivered within an Ig backbone *in vivo* results in induction of Th3 and Tr1 immunity.

27. The method of paragraph 1 wherein the T cell response is enhanced by co-stimulation with one of the following selected from the group consisting of anti-CD40mAb, recombinant IL-12 or synthetic dsRNA.

5 28. The method of paragraphs 1, 2, 3 or 4 wherein IL-2, IFN- γ and IL-4 are down-regulated in a dose dependent manner and IL-10 and TGF-beta are upregulated in a dose-dependent manner.

29. The method of paragraphs 1, 2, 3, or 4 wherein the peptide epitope is recNP and
10 induces NP-specific MHC class I-restricted T cell immunity consisting of IL-4 producing Tc2 cells.

30. The method of paragraph 1 further comprising the use of RNA motifs thereby resulting in a modified immune response.

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31. The method of paragraph 30 wherein the RNA motifs are dsRNA.

32. The method of paragraph 27 wherein the IgG1 and IgG2a antibody responses were increased and associated with an enhanced Th1 and Th2 response.

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33. The method of paragraph 2, 27 or 30 wherein the dsRNA was selected from the group consisting of pA:pU, pI:pC and pC:pG.

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34. The method of paragraphs 27 or 30 wherein the dsRNA is pA:pU and induced MHC class I-restricted Tc1 cells thereby producing IFN- γ .

35. The method of paragraphs 33 or 34 wherein the dsRNA are from 10 - 50Kd.

36. The method of paragraphs 2 or 30 wherein the RNA motifs are ssRNA selected
30 from the group consisting of p(A), p(C), p(G), p(I) and p(U).

84. The use of paragraph 81 wherein the dsRNA consists of bases selected from the group consisting of adenine, cytosine, uracil, guanine and inosine.

85. The use of paragraphs 81, 82 or 83 wherein the use enhances the Th1 and/or Tc1
5 response to the antigen.

86. The use of paragraphs 81, 82 or 83 wherein the use induces a Tc1 cell response to the antigen.

10 87. The use of paragraphs 81, 82 or 83 wherein the immune response includes an enhanced B cell response.

88. The use of paragraphs 81, 82 or 83 wherein the antigen is administered with additional antigen.

15 89. The use of paragraphs 81, 82 or 83 wherein the use induces expression of CXC and CC chemokines.

20 90. The use of paragraphs 81, 82 or 83 wherein the administering of dsRNA enhances T or B cell responses or both T and B cell responses by recruitment and activation of CD11b⁺ monocytes.

25 91. The use of paragraphs 81, 82 or 83 wherein the administering of dsRNA enhances T or B cell responses or both T and B cell responses by recruitment and activation of dendritic cells.

92. The use of paragraphs 81, 82 or 83 wherein the dsRNA compositions enhance an immune response by recruiting antigen presenting cells.

30 93. The use of paragraph 92 wherein the antigen presenting cell is a professional antigen presenting cell.

94. The use of paragraph 92 wherein the antigen presenting cell is a naive antigen presenting cell.

5 95. The use of paragraphs 81, 82 or 83 wherein the antigen is a non-infectious antigen and wherein the MHC Class 1 restricted T cells are cross-primed by the dsRNA.

96. The use of paragraphs 81, 82 or 83 wherein the composition and antigens are administered by one of the following selected from the group consisting of mucosal
10 administration, respiratory administration, intravenous administration, subcutaneous administration, and intramuscular administration.

97. The use of paragraph 81 wherein the antigen is administered in an immunoglobulin or portion thereof or in an immunoglobulin backbone.

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98. The use of paragraph 97 wherein the wherein the antigen is a peptide epitope.

99. A method of preventing high zone tolerance in a patient to an antigen comprising administering said antigen together with a dsRNA composition wherein the dsRNA
20 composition comprises at least one compound selected from the group consisting of poly-adenine, poly-uracil, poly-guanine, poly-cytosine, poly-inosine.

100. The method of paragraph 99 wherein the antigen is non-infectious.

25 101. The method of paragraph 99 wherein the antigen is administered in high doses or already present in the body.

102. The method of paragraphs 99, 100 or 101 wherein the dsRNA is selected from the group consisting of pA:pU and pI:pC.

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103. The method of paragraphs 99, 100, 101 or 102 wherein the method prevents B cell unresponsiveness.

104. A method of enhancing the immune system in a patient exposed to a pathogen
5 comprising the administration of dsRNA to the patient.

105. The method of paragraph 104 wherein the dsRNA is selected from the group consisting of pA:pU and pI:pC.

10 106. The method of paragraphs 104 or 105 wherein the dsRNA is administered to a patient in concentrations ranging from 100 ug/ml to 1 mg/ml.

107. The method of paragraphs 104, 105 or 106 wherein the pathogen is unknown.

15 108. The method of paragraphs 104, 105, 106 or 107 wherein the dsRNA is administered in a pharmaceutically acceptable carrier.

109. The method of paragraph 104 wherein a T cell response to the pathogen is enhanced.

20 110. A method of enhancing an immune response in a patient in need thereof comprising loading an antigen presenting cell by use of at least one peptide epitope of an antigen attached to an Ig backbone thereby forming an Ig-peptide complex or molecule and administering the Ig-peptide complex or molecule *in vivo* in conjunction with a
25 dsRNA motif wherein the epitope is effectively processed and presented by the MHC pathway of the antigen presenting cell resulting in effective loading of MHC molecules and thereby resulting in an effective secondary expansion of MHC molecules subsequent to *in vivo* exposure to the antigen.

30 111. The method of paragraph 110 wherein the MHC pathway is the MHC I pathway.

112. The method of paragraph 110 wherein the MHC pathway is the MHC II pathway.

113. The method of paragraph 111 wherein the method results in effective loading of MHC Class I molecules on the antigen presenting cell.

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114. The method of paragraph 112 wherein the method results in effective loading of MHC Class II molecules on the antigen presenting cell.

115. The method paragraphs 110, 111 or 112 wherein the dsRNA is pA:pU.

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116. The method of paragraphs 110, 111 or 113 wherein the method results in secondary expansion of MHC Class I restricted T cells.

117. The method of paragraph 115 wherein the antigen is a virus.

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118. The method of paragraph 117 wherein the virus is an influenza virus.

119. The method of paragraph 115 wherein the antigen is a tumor associated epitope.

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120. The method of paragraph 115 wherein the T cell is a cytotoxic T lymphocyte.

121. A method of generating an immune response to an antigen in a patient comprising:

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administering to the patient an immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of said antigen attached to said immunoglobulin or portion thereof and administering said immunoglobulin or portion thereof in conjunction with a dsRNA segment.

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122. The method of paragraph 121 wherein the immunoglobulin or portion thereof and said dsRNA segment are administered together.

123. The method of paragraph 121 wherein the immunoglobulin or portion thereof and said dsRNA segment are administered separately.

124. The method of paragraph 121 wherein said patient is human.

125. The method of paragraph 121 wherein upon administration of said immunoglobulin or portion thereof to said patient the immunoglobulin or portion thereof loads the antigen presenting cell by engagement with the antigen presenting cell's FcγR said peptide epitope is effectively processed and presented by the MHC I pathway of the antigen presenting cell resulting in effective loading of the MHC class I molecules.

126. The method of paragraph 121 wherein the peptide epitope is attached within the CDR region of the immunoglobulin or portion thereof.

127. The method of paragraph 121 wherein the immune response generates an effective T cell response to the antigen.

128. The method of paragraph 121 wherein the T cells are cytotoxic T lymphocytes.

129. The method of paragraph 121 wherein the dsRNA segment is selected from the group consisting of pA:pU and pI:pC.

130. The method of paragraph 121 wherein the peptide epitope is a T cell epitope.

131. The method of paragraph 121 wherein the peptide epitope is selected from the group consisting of influenza virus M1 or M2; hepatitis C virus NS3; hepatitis B virus core antigen; human papilloma virus HPV 18-E7, HPV 16 – E7, HPV 18 E6, HPV 16 E6; melanoma –gp100; MART-1; TRP-2; carcinoembryonic antigen precursor; Her – 2; tetanus toxin universal T helper epitope; HIV-1: reverse transcriptase; HIV1: gag; insulin precursor – human; human Gad 65; prostate tumor antigens; mucin 1; herpes simplex antigens; and, respiratory syncytial virus antigens.

132. The method of paragraph 121 wherein the immunoglobulin or portion and dsRNA segment thereof is administered by one of the methods selected from the group consisting of intravenous administration and bolus injection.
- 5 133. The method of paragraph 121 wherein the immunoglobulin or portion thereof and the dsRNA are administered in a pharmaceutically acceptable carrier.
134. The method of paragraph 121 wherein the method induces an effective memory response to the peptide epitope.

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Brief Description of the Drawings:

Fig. 1A shows (a) representation of natural IgG (light chain – heavy chain heterodimer); (B) antigen (Ag) derived peptide inserted within CDR (complementarity determining region) 3, 2, 1 or framework region; (C) VH (heavy chain, variable region) segment replaced with an antigen or fragment; (D) VH and CH1 segments replaced with antigen or antigen fragment;

Fig. 1B illustrates diagrammatically the IgG-peptide and Fc peptide;

Fig. 1C shows properties of selected human IgG backbone;

Fig. 1D shows the sequence of the constant region of the heavy chain as well as schematic depiction of a prospective construct;

Figs. 1E - 1M show the sequences of various antigens and epitopes discussed in the present application and which can be inserted into an immunoglobulin [sequences can be accessed on the internet at ncbi.nlm.nih.gov (add the proper address prefix: [http://www.](http://www.ncbi.nlm.nih.gov)) by searching the "proteins" section by use of the provided accession number. The content of this database is hereby incorporated by reference in its entirety.] ;

Figs. 2A - 2B show that while the injection of the peptide epitope in saline was not immunogenic, a similar dose of peptide used for *ex vivo* loading of APC effectively triggered a substantial immune response upon adoptive transfer;

Fig. 3 shows that delivery of epitope within Ig backbone considerably favored its stability in the systemic circulation;

Figs. 4A - 4B show that pre-incubation of peptide with serum resulted in decreased TcH activation;

Figs. 5A - 5B show that the relative efficiency of MHC-peptide complex formation greatly varied depending on the nature of antigen and APC;

5 Figs. 6A - 6B show that the peptide epitope within IgG backbone was more effective on a molar basis (1 order of magnitude) than the peptide alone in inducing TcH activation when handled by blood-derived APC;

10 Figs. 7A - 7B show that the use of oil-in-water adjuvant (incomplete Freund's adjuvant, IFA) only modestly enhanced the *in vivo* formation of MHC-peptide complexes on APC of lymph nodes but not the spleen or thymus;

Figs. 8A - 8D show that use of FcγR mediated delivery of peptides results in preferential formation of immunogenic MHC II - peptide complexes on CD11c+ and CD11b+ APC;

15 Figs. 9A - 9C show long lasting expression of peptide onto endogenous MHC II, on both DC (dendritic cells) and monocytes;

Fig. 10 shows that formation of MHC II - peptide complexes on dendritic cells and monocytes, subsequent to IgG mediated delivery of peptide epitope, is critically dependent on ITAM+ FcγR that encompass the gamma chain;

20 Fig. 11 shows that results show that the expression of the gamma chain of ITAM+ FcγR isoforms is necessary for the induction of T cell response to APC loaded with peptide within the IgG backbone;

Figs. 12A - 12D show that unexpectedly and in contrast with the potency / cell basis (Example 8), at the organism level, the CD11b⁺ monocytes have the highest impact on the immune response to a peptide epitope delivered within the IgG backbone;

25 Figs. 13A - 13B shows that FcγR-mediated delivery of a T cell epitope within the recombinant Ig backbone results in Th2 rather than Th1 response;

Fig. 14 shows that FcγR-mediated delivery of T cell epitope within recombinant Ig backbone results in Th2 rather than Th1 response;

30 Fig. 15 shows that a peptide epitope within the IgG backbone triggers a cellular response of Th2 profile that is enhanced but not switched by a conventional adjuvant (CFA);

Fig. 16 shows that peptide presentation by APC, subsequent to loading with antigen by using recombinant IgG as delivery platform, occurs in context of limited co-stimulation;

5 Figs. 17A-17B show that the activity of HA (110-120 hemagglutinin peptide) specific IL-4 producing T cells triggered by administration of recHA(I-Ed)-IgG is dependent on CD4 rather than CD8;

Fig. 18 shows that the IgG mediated delivery of T cell epitope has a profound and differential effect on the expansion and cytokine production by activated T cells: IL-2, IFN- γ and surprisingly IL-4, were down-regulated in a dose-related manner;

10 Figs. 19A – 19B show that in contrast to viral immunization with an influenza virus strain bearing the cognate peptide, Ig-mediated peptide delivery was ineffective in triggering cytotoxic response;

Figs. 20A – 20D show that co-administration of MBP and PLP epitopes by using recombinant IgG curbed the chronic progression of disease;

15 Fig. 21 summarizes the impact of IgG / Fc γ R-mediated delivery of epitopes on the T cell response, based on data provided in Examples 2-20;

Fig. 22 shows that shows that natural, non-infectious double stranded RNA produced during infection with influenza virus, has substantial effects on the specific immune response to a protein antigen;

20 Fig. 23A shows an extensive library of synthetic RNA motifs;

Figs. 23B – 23D show that different synthetic RNAs have an enhancing effect on the B and T cell response to a prototype protein antigen;

Figs. 24A - 24B show effects of selected RNA motifs on the innate immune response;

25 Fig. 25 shows that distinct RNA motifs bind to different receptors on antigen presenting cells;

Fig. 26 shows that distinct RNA motifs induce differential upregulation of chemokines;

30 Fig. 27 shows that the control of replication of influenza virus can be achieved by using selected synthetic RNA motifs;

Fig. 28 shows that selected synthetic RNA motifs pI:pC and pA:pU largely prevent high zone tolerance that is usually associated with administration of large amounts of purified protein;

Fig. 29 shows that selected synthetic RNA motifs effect on human monocytic
5 cells;

Figs. 30A – 30B show that non-tagged pA:pU, but not non-tagged pI:pC, was able to compete out the binding of tagged pA:pU to human THP-1 monocytic cells;

Fig. 31 shows the purification and fractionation steps of dsRNA;

Fig. 32 shows that lower molecular weight fractions of a selected synthetic RNA
10 compounds are endowed with different biological activity;

Fig. 33 shows that pI:pC but not pA:pU induced antibody response against itself, with a cross-reactive component against another RNA motif;

Figs. 34A – 34B show that co-use of selected synthetic RNAs promote effective induction of IL-2 and IFN-gamma subsequent to IgG mediated delivery of an MHC class
15 I-restricted epitope;

Fig 35 shows that *ex vivo* APC loading by recombinant IgG is more effective in formation of MHC class I-peptide complexes and generation of Tc response, compared to use of free peptide itself;

Fig. 36 show that IgG mediated delivery of a class I restricted epitope is most
20 effective in priming class I restricted Tc1 responses when co-administration of selected synthetic RNA was carried out;

Fig. 37 shows that effective priming of anti-viral cytotoxic T cells requires both effective *in vivo* loading of APC with class I restricted epitope delivered via IgG, together with appropriate instruction by selected synthetic RNA motif;

Fig. 38 shows that immunization with a recombinant IgG bearing a viral class I
25 restricted epitope together with selected synthetic dsRNA, resulted in priming of an immune response capable of limiting the replication of a virus subsequent to infectious challenge;

Fig. 39 describes the tumor models used for testing the efficiency of Ig-peptide-
30 based molecules;

Fig. 40 shows that both effective *in vivo* loading of APC with tumor associated antigen, together with simultaneous activation by selected synthetic RNA motifs, are necessary and sufficient for effective control of tumor growth and induction of tumor rejection;

5 Fig. 41 shows that both effective *in vivo* loading of APC with tumor associated antigen, together with simultaneous activation by selected synthetic RNA, can trigger an effective immune response to tumor-associated antigens;

Fig. 42 shows that tumor infiltrating lymphocytes displaying the T cell receptor marker TCR β acquired expression of the activation marker CD25 upon treatment with
10 recombinant immunoglobulin bearing tumor associated epitope, together with selected synthetic dsRNA motif;

Fig. 43 shows that the treated mice that successfully rejected the tumor developed Tc1 responses against the tumor-associated epitope on the therapeutic Ig, along with Tc2 immunity;

15 Fig. 44 shows that successful rejection of tumor induced by indicated treatment is followed by effective protection against subsequent challenge with the same tumor, indicating development of effective immune memory; and,

Figs. 45A - 45B show that the emerging immunity, subsequent to the indicated treatment that results in tumor rejection, protects against challenge with loss of antigen
20 variants and is associated with overall expansion of cytokine producing cells.

37. The method of paragraph 1 wherein the peptide-epitope is NP and further comprising the coadministration of dsRNA motifs thereby resulting in effective induction of IL-2 and IFN-gamma.

5 38. The method of paragraph 1 wherein the APC are loaded *ex vivo* resulting in the formation of MHC class I-peptide complexes and generation of a Tc response.

39. The method of paragraph 38 wherein the APC are administered to the patient by adoptive transfer.

10 40. The method of paragraph 38 wherein the formation of MHC class I-peptide complexes results in differentiation of Tc2 cells producing IL-4 but not IFN-gamma.

41. The method of paragraph 38 wherein further comprising the step of administering
15 RNA motifs thereby resulting in a broadening of the T cell profile to include IFN-gamma producing Tc1 cells.

42. A method of immunization of a patient comprised of loading an antigen presenting cell by use of at least one peptide epitope of an antigen attached to an Ig backbone or
20 portion thereof thereby forming an Ig-peptide molecule and administering to the patient *in vivo* the Ig-peptide molecule in conjunction with a dsRNA motif wherein the epitope is effectively processed and presented by the MHC I pathway resulting in effective loading of MHC class I molecules and thereby resulting in an effective secondary expansion of MHC class I-restricted T cells subsequent to *in vivo* exposure to the antigen.

25 43. The method of paragraph 42 wherein the antigen is a virus.

44. The method of paragraph 43 wherein the virus is the influenza virus.

30 45. The method of paragraph 42 wherein the peptide-epitope is recIgG-NP(Kd).

46. The method of paragraph 42 wherein the dsRNA is pA:pU.

47. The method of paragraph 42 wherein the T cells are cytotoxic T lymphocytes.

48. The method of paragraph 42 wherein the secondary expansion of MHC class I-
5 restricted T cells subsequent to *in vivo* exposure to the antigen is greater than
administration of the recombinant antigen in sterile saline only.

49. A method of controlling and treatment of a tumor after clinical diagnosis, by loading
an antigen presenting cell by use of at least one tumor associated T cell epitope attached
10 to an IgG backbone or portion thereof thereby forming an IgG-peptide molecule and
administering the Ig-peptide molecule *in vivo* in conjunction with dsRNA.

50. The method of paragraph 49 wherein the tumor associated T cell epitope is
effectively processed and presented by the MHC I pathway resulting in effective loading
15 of MHC class I molecules on the antigen presenting cell thereby resulting in an MHC
class I-peptide complex.

51. The method of paragraph 49 wherein the method results in an immune response to the
tumor associated T cell epitope and tumor rejection.

52. The method of paragraphs 49, 50 or 51 wherein the dsRNA is pA:pU.

53. The method of paragraph 49 wherein the Ig-G peptide complex and dsRNA are
administered repeatedly as an anti-tumor therapy.

54. The method of paragraph 49 wherein upon tumor rejection, Tc1 immunity is
developed against the tumor associated epitope.

55. The method of paragraph 49 where upon administration of IgG-peptide and dsRNA,
30 Tc2 immunity is developed against the tumor associated epitope.

56. The method of paragraph 49 wherein the method further induces an effective memory response to the same tumor associated epitope.

57. The method of paragraph 49 wherein the method results in continued immunity to tumor cell variants.

58. The method of paragraphs 49, 50, 51, 52, 53, 54, 55, 56, or 57 wherein the tumor associated T cell epitope is selected from the group consisting of melanoma -gp100, MART-1, TRP-2, carcinoembryonic antigen precursor XP 064845/NCB1, Her -2, prostate tumor antigens, and MUC 1.

59. A recombinant human Ig molecule or portion thereof capable of binding to an FcγR of an APC, comprising of a CH₃ region adjacent to a CH₂ region whereby a hinge region attaches an antigen to the CH₂ region wherein the antigen has an oligo-glycine linker attached to the hinge region.

60. The recombinant human Ig molecule of paragraph 59 whereby the antigen has a flanking sequence extending therefrom followed by a leader.

61. The recombinant human Ig molecule of paragraph 59 wherein the human Ig molecule is an IgG molecule.

62. The recombinant human Ig molecule of paragraph 59 wherein the antigen is a viral or tumor antigen.

63. The recombinant human Ig molecule of paragraph 59 wherein the amino acid sequence of the CH₃ region is:

GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP

GK and conservatively modified variants thereof.[Seq. I.D. No. 1].

64. The recombinant human Ig molecule of paragraph 59 wherein the amino acid sequence of the CH₂ region is: APELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL NGKEYKCKVFNKALPAPIEKTISKAK and conservatively modified variants thereof.

[Seq. I.D. No. 2].

65. The recombinant human Ig molecule of paragraph 59 wherein the amino acid sequence of the hinge region is: EPKSCDKTHTCPPCP and conservatively modified variants thereof. [Seq. I.D. No. 3].

66. The recombinant human Ig molecule of paragraph 53 wherein the amino acid sequence of the flanking sequence is: QVQLQ and conservatively modified variants thereof. [Seq. I.D. No. 4].

67. A composition for enhancing an immune response to an antigen wherein the composition is a polynucleotide wherein the polynucleotide is made up of compounds selected from the group consisting of adenine, uracil, guanine, cytosine and inosine.

68. The composition of paragraph 67 wherein the polynucleotide is dsRNA.

69. The composition of paragraph 68 wherein the dsRNA is selected from the group consisting of pA:pU and pI:pC.

70. The composition of paragraph 69 wherein the dsRNA is pA:pU and wherein some of the adenine and uracil is occasionally replaced by guanine, cytosine or inosine along the polynucleotide chain.

71. The composition of paragraph 69 wherein the antigen is a virus.

72. The composition of paragraph 69 wherein the antigen is attached to an immunoglobulin or portion thereof and administered *in vivo*.

73. The composition of paragraph 72 wherein the antigen is protein or a peptide.

74. The composition of paragraphs 67, 68, 69 or 70 wherein the antigen is a tumor
5 associated epitope.

75. The composition of paragraph 74 wherein the antigen is a T cell epitope.

76. The composition of paragraphs 67, 68, 69 or 70 wherein the dsRNA is administered
10 together with said antigen.

77. The composition of paragraph 67 wherein the polynucleotide is dsRNA and is
coadministered with the antigen.

15 78. The composition of paragraph 67 wherein the antigen is already present in the body.

79. The composition of paragraph 67 wherein the antigen is administered in a
pharmaceutically acceptable carrier.

20 80. Use of dsRNA in the manufacture of a medicament for enhancing an immune
response to an antigen in a patient, comprising administering said dsRNA to a patient in
conjunction with said antigen.

81. The use of paragraph 80 wherein an epitope of said antigen is delivered to the patient
25 in an immunoglobulin or portion thereof.

82. The use of paragraphs 80 or 81 wherein the dsRNA is comprised of pA:pU.

83. The use of paragraphs 80 or 81 wherein the dsRNA is comprised of pI:pC.
30

Detailed Description of the Invention**Definitions:**

5

The following definitions are intended to act as a guide and are not to be considered limiting of terms found throughout the specification:

10

adjuvant - a substance that enhances the adaptive arm of the immune response to an antigen;

adoptive transfer - transfer of a cell population from one animal to another of the same haplotype;

15

antigen - a molecule that can be specifically recognized by the adaptive elements of the immune system (B cells, T cells or both);

antigen presenting cell - heterogeneous population of leukocytes with very efficient immunostimulatory capacity;

20

BALB/C mouse - Widely distributed and among the most widely used inbred mouse strains;

25

B cell - a type of lymphocyte developed in the bone marrow. Each B cell encodes a surface receptor specific for a particular antigen. Upon recognition of a specific antigen, B cells multiply and produce large amounts of antibodies which in turn bind to the antigen which activated the B cell;

30

B cell unresponsiveness - antigen-specific lack of response by B cell;

CDR - Complementarity Determining Region; hypervariable regions in an immunoglobulin which create the antigen binding site. There are three CDR regions: CDR1, CDR2 and CDR3;

chemokines - a group of at least 25 small cytokines, all of which bind to heparin;

5 complete Freund's adjuvant - an oil-in-water emulsion containing mycobacterial cell wall components;

cross primed - antigen presenting cells that have acquired antigens from infected tissues and then present them to cognate T cells;

10

Dendritic Cells - A subtype of antigen presenting cells (i.e. CD11c+);

downregulation - decreasing the expression or activity of a particular compound or effect;

15

epitope - parts of an antigen which contact the antigen binding site of the antibody or T cell receptor;

20 Fc γ R - Ig receptors on cell surfaces of which there are three recognized groups: Fc γ RI (CD64), Fc γ RII (CD32) and Fc γ RIII (CD16);

heterodimer - dimeric protein consisting of 2 different protein sequences;

25 high zone tolerance - a state of unresponsiveness specific to a particular antigen that is induced upon challenge with a high concentration of said antigen;

IL-2 - refers to interleukin - 2;

30 IL-4 - refers to interleukin - 4;

Immunoglobulin - a group of glycoproteins present in the serum and tissue fluids of all mammals and are located on the surface of B cells and serve as antibodies free in the blood or lymph. There are five classes of immunoglobulins: IgG (70 - 75%), IgM (10%),

IgA (15 - 20%), IgD (>1%) and IgE (found on basophils and mast cells in all individuals). IgG has four human subclasses (IgG1, IgG2, IgG3 and IgG4);

Immunoglobulin backbone - refers to an immunoglobulin molecule or portion thereof wherein at least one CDR region is able to receive an inserted peptide epitope;

immunoglobulin isotype switching - stimulation of B cells to switch production from one immunoglobulin isotype to another;

incomplete Freund's adjuvant - an oil-in-water emulsion not containing mycobacterial cell wall components.;

innate immunity - The innate immune system provides broad relatively nonspecific host defenses that lack antigenic specificity but have the ability to guide acquired immunity. Among the cells types involved are dendritic cells and macrophages;

intraperitoneally- within peritoneal cavity;

intravenously - within vasculature;

isoforms - different glycosylation, phosphorylation, deamidation and other posttranslational modifications of proteins;

ITAM - immunoreceptor tyrosine-based activation motifs;

ITIM - immunoreceptor tyrosine-based inhibitory motifs;

macrophages - Any mononuclear, actively phagocytic cell arising from monocytic stem cells in the bone marrow;

MHC - refers to the Major Histocompatibility Complex;

5 modified immune response - enhanced or diminished immune response;

monocytes - Mononuclear leukocytes found in lymph nodes, spleen, bone marrow and loose connective tissue;

10 naive - non-differentiated, non-activated cell;

peptide - a compound consisting of two or more amino acids joined together by a peptide bond;

15

polynucleotide - a polymer of nucleotides;

professional antigen presenting cell - mature, able to present antigenic epitope;

20

recruitment - attraction of a cell population to inflammatory site;

secondary expansion - immune response which follows a second or subsequent encounter

25

with a particular antigen;

self-antigens - antigens that are derived from the host;

30

subcutaneously - beneath the skin;

Tc1 immunity - Cytotoxic T cell type 1, CD 8+;

35

Th1 cells - T helper 1 cells which are involved in cell mediated inflammatory reactions, identified by production of $IFN\gamma$, $TNF\beta$ and IL-2;

Th2 cells - T helper 2 cells which encourage production of antibodies and are identified by production of IL-4 and IL-5;

Th3 cells - T helper regulatory cell, known to produce transforming growth factor (TGF)-beta;

TR1 cells - T regulatory cell, known to produce interleukin 10; and,

upregulation - enhancement of expression or activity of a particular compound or effect;

Materials and Methods

For selective *in vivo* loading of antigen presenting cell subsets, the use of compounds described schematically in the Figure 1A are used: (A) representation of natural IgG (light chain – heavy chain heterodimer); (B) antigen (Ag) derived peptide inserted within CDR 3, 2, 1 or framework region; (C) VH segment replaced with an antigen or fragment; and, (D) VH and CH1 segments replaced with antigen or antigen fragment. This type of molecules are engineered using methods known in the art and as stated as follows:

Construction of model recombinant IgG.

Polymerase chain reaction (PCR) mutagenesis was used to replace the CDR3 region of VH chain with the stated epitopes. Briefly, a pUC19 plasmid harboring the 5.5-kb EcoRI fragment carrying the VH gene of the murine anti-arsonate antibody, 91A3, was used as template DNA in two PCRs to delete the diversity segment (D) of the complementarity-determining region 3' (CDR3) loop and inserted DNA fragments encoding various antigen epitopes. These chimeric VH and as well as wild type VH genes were then ligated with Ig gamma 1 heavy chain constant region within the plasmid pSV2ΔHgptDNSVH-hCgamma1 from which the EcoRI dansyl (dns)-conjugated VH gene was cut out. The sequences of VH and inserted epitopes were confirmed by DNA sequencing. To express these chimeric IgGs with murine 91A3 VH-human C gamma1 heavy chain genes and a mouse-human chimeric k light chain gene, an 8-kb BamHI fragment encoding the entire murine 91A3 kappa light chain gene was subcloned into the

BamHI site of pUC19 plasmid. Subsequently, a HindIII fragment with the kappa light chain promoter and the V kappa region coding sequences was cut out from this plasmid and subcloned into the HindIII site of pSV184ΔHneoDNSVk-hCk upstream of the gene encoding a human k light chain C region (Ck) from which the dns-conjugated Vk (dnsVk) had been excised. This plasmid, which will encode a murine 91A3 Vk-human Ck light chain, is designated pSV184Δhneo91A3Vk-hCk.

10 Construction of human recombinant IgG.

The human IgG backbone was obtained from IgGA1 myeloma cell line by RT-PCR. The recombinant human IgG was cloned by inserting the stated epitopes to replace the CDR2 or CDR3 regions of the human IgG1 backbone. Briefly, T cell epitopes were created by PCR mutagenesis and subcloned into the CDR2/CDR3 region. The recombinant heavy chains were then subcloned into pMG vector (Invivogen, San Diego, CA) by BamHI and XbaI sites. The heavy chain expression was controlled by the hCMV promoter. In parallel, the human kappa light chain was subcloned into the pMG vector by StuI and NheI sites. The expression of the light chain was controlled by an EF-1 alpha and HTLV-1 LTR hybrid promoter. The double expression vector carrying both the recombinant heavy chain and light chain were then transfected into expression cell lines.

The Fc-peptides were constructed by cutting off the VH and CH1 fragment and replacing it with stated viral or tumor antigens (8-150 Aas). Briefly, the human IgG1 heavy chain was subcloned into pCDNA3 vector by EcoRI and XhoI sites. Then the stated antigens are inserted between the leader sequence and hinge region of IgG1 by PCR mutagenesis. To increase the flexibility of the fused antigens, an oligo-glycine linker (5 glycines) was added after the antigen. The expression of human IgG recombinant molecules can be performed by using either one of the strategies displayed in Figure 1B.

The human IgG backbone has been selected rationally, based on the ability to bind to FcγR, complement and cytokine activation in various states. Properties of selected human IgG backbone are shown in the Figure 1C and the sequence of the constant region

of the heavy chain as well as the schematic depiction of a prospective construct, is shown in Figure 1D.

Epitopes used for model recombinant IgG are shown in Figure 1E (mouse MHC class II-restricted HA epitope and mouse MHC class I restricted NP epitope). The nomenclature of recombinant constructs is recIgG-epitope (HA or NP)- restriction element (I-Ed or Kd, respectively). In short, they may be referred to as IgHA or IgNP. Model molecules comprising defined mouse self epitopes (MBP or PLP derived) were similarly constructed. The sequence of the variable region of the heavy chain of anti-arsonate antibody used as the backbone has been depicted in Figure 1E and the technology is well known in the art (Zaghoulani et al., Science 1993 Jan 8;259(5092):224-7) the contents of which is hereby incorporated by reference.

In Figures 1E-1M, examples of antigens and epitopes (in bold) are provided that could be inserted (larger parts up to 150 AA spanning one or multiple epitopes) or attached to the backbone. Such constructs comprising the shown antigens / epitopes may be used as drugs against infectious or tumoral diseases. In Figure 1I there is the HLA-A2 anchor motif displayed, that allows the prediction of location of potentially therapeutic cytotoxic epitopes in any protein, facilitating the selection of the antigen fragment to be used in the recombinant immunoglobulin.

In Figure 1J, examples of "universal" T helper epitopes (Kumar et al. J Immunol 1992 Mar 1;148(5):1499-505) are provided, both dominant and promiscuous from the point of view of MHC restriction, that could be used for construction of composite molecules for the purpose of inducing or enhancing immunity to MHC class I-restricted epitopes, using compounds such as:

[antigen fragment]-[universal Th epitope]-Fc(IgG).

Examples of such constructs are schematically represented in Figure 1K (bottom).

In Figure 1K top, examples of human self antigens with epitopes bolded are shown, that could be used to generate recombinant IgG molecules against autoimmune / inflammatory disorders.

In Figure 1L and 1M other antigen sequences that could be used for the construction of above mentioned immunoglobulin constructs are shown. The antigen

fragments of interest could be defined by using methods to predict MHC class I epitopes (Lim et al., Mol Immunol. 1996 Feb;33[2]:221-30).

Production of recombinant IgG

5 The SP2/0 cell line (American Type Culture Collection) is used for the production of all the recombinant IgGs (rIgG) discussed in this patent application. Stable expressing cell lines (i.e. transfectomas) were produced using a double transfection protocol with plasmids encoding the heavy and light chains of an anti-arsenate mouse IgG. Each transfectoma differs only in the sequence of the CDR3 region of the heavy chain.

10 Methods for growing the cell lines as well as producing the different purified rIgG used in the experiments reported in this application are identical in all cases.

 The SP2/0 transfectomas were initially grown in Quantum Yield media (BD Biosciences) supplemented with 5 % (v/v) heat-inactivated fetal bovine serum, 0.5 mg/mM gentamicin and 2.5 µg/mL Fugizone. Cultures were maintained at 37°C in a

15 humidified CO2 incubator. Efforts were made to adapt each of the cell lines to growth in different commercially available serum-free medias (Lymphocyte Growth Media 2, Clonetics; Cell MAb Growth Media Serum Free, BD Biosciences; Animal Component Free Cell Media, BD Biosciences). Each of the serum-free medias was supplemented with antibiotics as above. Culture media containing secreted IgG was produced from each

20 media noted above. No difference in the IgGs produced in the different medias was observed over the course of this work (molecular weight analysis by SDS PAGE [see below], ELISPOT assays, and immune responses in mice).

 The amount of secreted rIgG was quantitated using an ELISA: capture antibody was a goat anti-mouse IgG (Sigma) and secondary antibody was an anti-mouse IgG HRP

25 conjugate (Sigma). Purified mouse IgG (Sigma) was used as a standard.

 Four different methods have been used to produce media containing the different rIgGs (i.e. conditioned media, "CM"): flasks, stirred vessels, packed bed bioreactors (New Brunswick Cellagen), CELLline flasks (BD Biosciences). In the case of CM produced in flasks, the cells were fed and/or harvested twice a week and maintained at

30 least 50% viability, but viability was generally greater than 70%. Collected media was filtered and held at 4 C. Stirred vessels (1 L) were seeded at 10^6 cells per mL in 200 mL

starting volume. Media was added weekly to keep the cell number between 10^7 and 10^6 per mL until 800 mL of total volume was reached. At this point cell viability was determined (typically greater than 80%), and the run was continued until such time that the viability fell below 50%. Media was then collected and sterile filtered to remove cells and held at 4°C. For the packed bed bioreactors: each unit was seeded with approximately 10^8 cells in 400 mL of media; maintained in a CO₂ incubator at 37°C with constant stirring; media was changed every 3-4 days and CM was filtered as above; production of rIgGs in the CM was monitored with ELISA. Bioreactor runs were continued until production of rIgGs began to decline or the vessel became contaminated. The 1 L CELLline flasks were used according to manufacturer's instructions: each flask was seeded with 10^7 to 10^8 cells in a total volume 40 mL in the cell compartment; 1 L of media was added to the feed compartment; CM was harvested from the cell chamber after 2 to 3 weeks, or when viability of the cells fell below 20%.

Purification of rIgG

The rIgGs produced by the above methods were purified by one of two methods. For CM that contained FBS, an anti-mouse IgG immunoaffinity resin was used. The immunoaffinity resin was synthesized using the following protocol: 10 mL of cyanogen bromide-activated Sepharose 4B (Sigma) was washed with 1 mM HCl as per manufacturer's instructions; 10-20 mg of goat anti-mouse IgG (Sigma) was dissolved in coupling buffer (0.1 M sodium carbonate [pH 8.4]/0.5 M NaCl) at a concentration of 2 mg/mL; the IgG solution was added to the washed resin, and the slurry was mixed end-over-end at room temperature; the extent of coupling was monitored using the Bradford assay to determine the amount of remaining soluble IgG; the coupling was quenched by addition of ethanolamine to a final concentration of 10 mM when the amount of soluble IgG was less than 10% of the starting concentration (approximately 45 minutes). The immunoaffinity resin was then washed with the following buffers: PBS, 10 mM glycine (pH 2.4), 20 mM Tris/ 1 M NaCl (pH 8.0), PBS. The resin was stored at 4°C in PBS. The protocol for purifying rIgG with this resin was initiated by passing CM through the column at 1 to 2 mL/min. The resin was then washed free of nonbound protein using the following protocol: 100 mL PBS/0.5M NaCl followed by 50 mL 1 mM Tris (pH 8).

Fractions were monitored for protein using the Bradford assay. Specifically bound rIgG was eluted with a low pH buffer (5 mM glycine (pH 2.4)/0.5 M NaCl). The eluted protein was collected and held at 4°C for further processing (see below).

The rIgG produced in serum-free culture media was purified using Protein A affinity chromatography. Typically, a 5 mL rProtein A column (HiTrap rProtein A FF from Amersham Pharmacia Biotech) was equilibrated with PBS and the sample was run through the column at 2 mL/min using a FPLC unit (Pharmacia). The resin was washed free of nonspecifically bound protein with PBS, followed by 20 mM Tris (pH 8.0)/1 M NaCl, then water. The specifically bound rIgG was eluted with 1 mM glycine (pH 2.4).
10 The eluted peak was collected and held at 4 C for further processing.

Generally, the rIgG fractions were pooled and concentrated using Centricon ultrafiltration units (Amicon) to a final concentration of 1 to 4 mg/mL (Bradford assay with IgG as standard). The concentrated fraction was then dialyzed into 1 mM glycine (pH 2.4), the final concentration determined by A_{280} using an extinction coefficient of 1.4
15 for a 1 mg/mL IgG solution, and aliquoted into 100 μ l fractions that were stored in the -80°C freezer. The purified rIgGs were analyzed for structural integrity and purity by SDS gel electrophoresis. The gels were stained with Coomassie blue (Pierce Chemical). In all cases the rIgGs used in the reported experiments displayed their expected molecular weight (reduced and nonreduced) as compared to protein standards and control IgG.
20 Generally, the purified rIgG was greater than 95% pure as determined by visual inspection of the stained bands relative to the bands of known amounts of control IgG run on the same gel.

RNA segments

25 The double stranded RNA (dsRNA) or single stranded RNA (ssRNA) segments of the present invention can be made according to the following method (and are available commercially): 1) ssRNA: The polynucleotides (polyA, polyU) are enzymatically prepared, using nucleotides and polynucleotide-phosphorylase, with no animal-sourced material entering into its preparation process. 2) dsRNA: Annealing of
30 polyadenylic acid (polyA or pA) with polyuridylic acid (polyU or pU).

In general, the dsRNA and ssRNA of the present invention are homopolymers with, in the case of dsRNA, a single base or nucleotide (e.g., adenine) consistently forming one strand with its complement consistently forming the other strand. In the case of ssRNA, the single strand is consistently made of the same nucleotide. However, it is within the scope of the invention to use dsRNA or ssRNA compositions that are made up of mixed nucleotides (and without or without their complements in the case of dsRNA). For example, a polyA:polyU dsRNA segment with occasional substitution by an a non-complementary nucleotide (e.g., guanine, cytosine or inosine). The dsRNA and ssRNA compositions of the present invention are comprised of the bases/nucleotides adenine (A), guanine (G), cytosine (C), uracil (U) and inosine (I) and could also be comprised of a small percentage of the DNA base thymine (T). The RNA compositions in Table I and Figure 8A is descriptive of various RNA compositions used in the Examples. The RNA compositions of the present invention were prepared and purified according to Example 30.

The various RNA strands used in the present invention are generally between 100 - 2000 base pairs in length but may be between 1 - 20, 20 - 40, 40 - 60, 60 - 80, 80 - 100, 1 - 100, 100 - 200, 200 - 300, 300 - 400, 400 - 500, 500 - 600, 600 - 700, 800 - 900, 1000 - 1100, 1100 - 1200, 1200 - 1300, 1300 - 1400, 1400 - 1500, 1500 - 1600, 1600 - 1700, 1700 - 1800, 1800 - 1900, 1900 - 2000, 2000 - 2100, 2100 - 2200, 2300 - 2400, 2400 - 2500, 2500 - 3000, 3000 - 4000, 4000 - 5000, 5000 - 10,000 base pairs and greater than 10,000 base pairs in length and/or mixtures thereof.

Example 1 shows that a significant factor limiting the activity of peptides that encompass T cell epitopes is the poor pharmacokinetics resulting in reduced *in vivo* loading of APC.

Antigen presenting cells ("APCs") from 1 naïve BALB/c mouse were obtained from splenic tissue. Following washing, three million APC were incubated with 13.5nM HA 110-120 peptide for 3 hours at 37°C, in 1 ml of HL-1 medium. The cells were washed, divided into three equal inoculi and injected (1/2 subcutaneously + 1/2 intraperitoneally) into 3 naïve BALB/c mice. The mice were sacrificed 2 weeks later and the immune response measured against HA 110-120 peptide, by ELISPOT analysis as

follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4 μ g/ml for anti-IL2 and anti-IL4, and 8 μ g/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μ l/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200 μ l/well of DMEM complete containing FBS, for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 20 μ g /ml HA 110-120 peptide or just with media, to assess the background.

Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, plates were washed 5 times with PBS-tween20 0.05% (washing buffer), and incubated with 100 μ l /well of biotinylated anti-cytokine Abs, 2 μ g /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C. The next day, the plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. Plates were then allowed to dry at room temperature for 24 hours. The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). In parallel, 3 naïve BALB/c mice were each injected with 4.5nM of HA peptide in sterile PBS, half of it administered subcutaneously and half of it intraperitoneally. The mice were sacrificed 2 weeks later and the T cell response characterized as above, by ELISPOT analysis.

In Figure 2(A), the experimental protocol is described. In Figure 2(B), the results of the experiment are shown: they were expressed as number of IFN- γ , IL-2 and IL-4 spot forming colonies / spleen, after the subtraction of the background (mean \pm SEM). "HA-APC" corresponds to antigen presenting cells (dendritic cells) loaded *ex vivo* prior to adoptive transfer. "HA" corresponds to peptide directly injected into animals.

The results described in the Figs. 2A - 2B show that while the injection of the peptide epitope in saline was not immunogenic, a similar dose of peptide used for *ex vivo* loading of APC effectively triggered a substantial immune response upon adoptive transfer. This shows that if directly injected, the peptide does not effectively reach APC, a prerequisite for effective induction of an immune response.

Example 2 demonstrates that incorporation of a peptide epitope within the IgG ameliorated its pharmacokinetics profile.

5 BALB/c *Scid* mice (3/group) were injected intravenously with 60nM of SFERFEIFPKE ("HA") [Seq. I.D. No. 5] peptide or 2.4nM of recHA (I-Ed)-IgG ("Ig-HA") and blood was harvested at various intervals. Serum was immediately separated and promptly frozen at -70°C. Later, the serum samples were incubated with 2X10⁴ cells/well/50µl HA-specific T cell hybridoma (TcH) and 1x10⁴ cells/well/50µl M12 B
10 cell lymphoma APC, in serum free HL-1 medium at 37°C and 5% CO₂ for 24 hours. The next day the plate was centrifuged for 15min/4°C/1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4°C/1500RPM. Fixing solution was flicked off the plate, cells washed once with
15 PBS 200 µl/well, centrifuging the plate for 3min/4°C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37°C with 200µl/well of the X-gal substrate freshly prepared as follows: 200µl of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the
20 microscope.

The activation of TcH was represented as function of time post-injection. The epitope could be detected in the blood only in the case of mice injected with recHA(I-Ed)-IgG, for an interval of about one day. In contrast, the HA peptide injected as is, was not detected in the periphery despite being used in large molar excess (25 fold).

25 Thus, the results described in the Fig. 3 show that delivery of epitope within Ig backbone considerably favored its stability in the systemic circulation.

Example 3 shows that a peptide encompassing a T cell epitope is ineffectively presented by APC to specific T cells in the presence of serum and this is corrected by incorporation of the peptide epitope within the IgG backbone
30

Figure 4(A) shows the detrimental effect of serum on the presentation of a T cell epitope peptide: M12 B cell lymphoma APC were incubated with TcH in the presence of

various amounts of SFERFEIFPKE (HA) peptide in serum-free HL-1 medium ("HA+HL-1") or HL-1 medium supplemented with 20% mouse serum from BALB/c *scid* mice ("HA+serum"). The number of cells incubated was 2×10^4 M12 and 1×10^4 TcH / 100 μ l of HL-1 medium supplemented or not with serum. The next day the plate was

5 centrifuged for 15min/4⁰C/1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4⁰C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 μ l /well, centrifuging the plate for 3min/4⁰C/1500RPM. PBS was flicked off the plate and cells were incubated

10 overnight at 37⁰ C with 200 μ l/well of the X-gal substrate freshly prepared as follows: 200 μ l of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope.

The serum negatively interfered with the formation and / or presentation of

15 immunogenic MHC-peptide complexes.

Figure 4B: the serum negatively interfered with the formation and / or presentation of immunogenic MHC-peptide complexes.

This phenomenon was further studied by sequential incubation of peptide ("HA peptide") or recHA (I-Ed)-IgG ("IgHA") first with APC or serum, followed by addition

20 after 1 hour of TcH and serum, or APC and TcH, respectively. Control corresponds to cells incubated with antigens in the absence of added serum ("Ctrl"). The number of cells incubated was 2×10^4 M12 and 1×10^4 TcH / 100 μ l of HL-1 medium supplemented or not with serum. The next day the plate was centrifuged for 15min/4⁰C/1500RPM, then the

25 supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4⁰C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 μ l /well, centrifuging the plate for 3min/4⁰C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37⁰ C with 200 μ l/well of the X-gal substrate

30 freshly prepared as follows: 200 μ l of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide,

2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope.

The results were represented as percentage of activated T cells (beta-gal⁺ TcH) / well at concentrations of 2μg/ml of recHA (I-E^d)-IgG ("IgHA") or 40μg/ ml of HA peptide (1,000 molar excess relative to the recombinant Ig).

The results described in the Fig. 4 show that pre-incubation of peptide with serum resulted in decreased TcH activation. Addition of serum after APC pulsing did not have an effect on TcH activation. In contrast, the formation of MHC-peptide complexes was not impaired by serum when the recombinant immunoglobulin carrying the peptide was used instead of the peptide alone.

Example 4 shows that incorporation of a T cell peptide epitope within an IgG backbone improves its presentation to specific T cells by APC, with a rate depending on the nature of APC.

As shown in Figure 5A, *ex vivo* formation of MHC-peptide complexes on antigen presenting cells (APCs) from spleen was measured as follows: splenic APC were isolated by magnetic sorting using anti-MHC II antibodies. Separation by using magnetic beads coupled with anti-MHC II was carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as follows: spleens were processed to single cell suspension, red blood cells lysed, then cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative fraction runs through. After removal of the column from the magnetic field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in HL1 complete media and they were incubated with specific T cell hybridoma recognizing I-E^d+SFERFEIFPKE overnight, in the presence of various amounts of SFERFEIFPKE ("HA") peptide or recHA(I-E^d)-IgG ("IgHA"). Per well, 2x10⁴ APC were incubated with 1x10⁴ TcH. Next day the plate was centrifuged for 15min/4°C/1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and

the plate was again centrifuged for 3min/4°C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 µl /well, centrifuging the plate for 3min/4°C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37°C with 200µl/well of the X-gal substrate freshly prepared as follows: 200µl of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope. The number of activated TcH was quantified and the results expressed as activation versus molar amount of epitope.

- 10 (B) A protocol similar to that described above has been applied to M12 B cell lymphoma APC.

Thus, the results described in the Figure 5B show that the relative efficiency of MHC-peptide complex formation greatly varied depending on the nature of antigen and APC. On a molar basis, the peptide epitope within the IgG backbone was 10 times more effectively handled by MHC II+ APC from lymphoid organs and 1000 times more effectively handled by transformed B cell lymphoma cells, as compared to the free peptide itself. Thus, the cellular handling of the epitope and formation of MHC-peptide complexes subsequent to delivery within IgG, greatly varies with the nature of APC.

- 20 **Example 5 shows that FcγR-mediated delivery of a peptide encompassing a T cell epitope results in more effective cellular handling and presentation by cell populations (peripheral blood white cell) containing reduced numbers of professional APC.**

- 25 (A) To quantify the APC, peripheral blood mononuclear cells (PBMC) were separated by Ficoll gradient centrifugation from BALB/c mice and FACS analysis for expression of CD11c, CD11b and B220 was carried out. The results are represented in Figure 6A as percentage of APC and T cells in blood versus a prototype secondary lymphoid organ (spleen). The number of professional APC such as CD11c+ cells is tremendously (2 logs) decreased in blood as compared to spleen. B220+ and CD11b+ cells were decreased as well (1 order of magnitude). The following materials and methods were used.
- 30

Materials:

Ficoll: Ficoll-hypaque (1.077, Amersham, cat# 17-1440-02)

Antibodies: CD11b cat#01715A, CD11c cat# 557401, B220 cat#01125A, all PE conjugated (BD PharMingen)

5 Flow Cytometer: FACSCalibur, Becton Dickinson

FACS Buffer: PBS, 1% FCS, 0.1% sodium azide.

Methods:

1. Animal blood was harvested and mononuclear cells were separated by Ficoll
10 gradient separation.
2. Cells were suspended and labeled with fluorescently-tagged anti-mouse CD-11c,
CD11b or B220 at 2 ug/ml for 20 minutes on ice
3. Cells were washed once and resuspended in 300 ul of FACS buffer
4. Flow cytometric analysis was carried out to determine fractions of total cell
15 population which labeled with each specific antibody

(B) PBMC were used as APC with SFERFEIFPKE (HA)-specific TcH, in the presence of cognate peptide or recHA (I-Ed)-IgG. The cells were co-incubated for 24 hours (2×10^4 APC + 1×10^4 TcH). The next day the plate was centrifuged for 15min/4C/1500RPM, then
20 the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4⁰C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 μ l /well, centrifuging the plate for 3min/4⁰C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37⁰C with 200 μ l/well of the X-gal substrate
25 freshly prepared as follows: 200 μ l of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope. The results are expressed as number of activated TcH / well, at different molar concentrations of epitope.

30 The results described in the Figures 6A - 6B show that the peptide epitope within IgG backbone was more effective on a molar basis (1 order of magnitude) than the

peptide alone in inducing TcH activation when handled by blood-derived APC, suggesting that in suboptimal conditions associated with limiting numbers of professional APC, the Ig backbone greatly facilitates the creation of MHC-peptide complexes.

- 5 **Example 6 shows that delivery of a T cell epitope within IgG backbone dramatically improves the loading and presentation of epitope by APC in the secondary (draining lymph nodes + spleen) but not central lymphoid organs. The emulsification of the peptide epitope in IFA or increase of dose 100 fold could not reproduce the same degree of loading. Thus, epitope insertion within the IgG backbone removes limiting**
10 **factors associated with peptide-based strategy, that cannot be otherwise compensated by dose escalation or depot effect.**

- Assessment of *in vivo* formation of MHC-peptide complexes and a comparison with peptide in saline or standard oil-in-water emulsion were carried out in I-Ed⁺
15 BALB/c mice. BALB/c mice were treated with recHA (I-Ed)-IgG, peptide in saline or peptide emulsified in incomplete Freund's adjuvant (IFA), by subcutaneous and intraperitoneal injection (doses depicted in Figure 7B). At 24 hours, the local (mesenteric) lymphoid nodes (LN), spleen and thymus were harvested, single cell suspensions were made, red blood cells lysed from the spleens, LN and thymus were
20 collagenase digested. All cells were washed, counted and incubated with TcH recognizing I-Ed+SFERFEIFPKE (MHC class II-HA) complexes. The number of TcH was 1×10^4 / well. The formation of such MHC – peptide complexes was evaluated by titrating the number of APC with constant number of TcH and measuring TcH activation after overnight incubation. The next day the plate was centrifuged for
25 15min/4⁰C/1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4⁰C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 μ l /well, centrifuging the plate for 3min/4⁰C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at
30 37⁰ C with 200 μ l/well of the X-gal substrate freshly prepared as follows: 200 μ l of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope.

The data are expressed as TcH activation versus APC number (Figure 7A) and as estimated percentage of APC expressing MHC-peptide complexes (Figure 7B), based on *in vitro* standard curve obtained as depicted in the previous Examples, 5 and 6.

The data presented in the Figures 7A - 7B show that the use of oil-in-water adjuvant (IFA) modestly enhanced the *in vivo* formation of MHC-peptide complexes on APC of lymph nodes but not spleen or thymus. Substantial dose escalation of peptide in saline or in emulsion is not paralleled by proportional enhancement in the generation of loaded APC and/or MHC - peptide complexes on APC *in vivo*. In contrast, use of peptide within Ig backbone enhances the formation of MHC peptide complexes considerably, on APC from secondary lymphoid organs such as lymph nodes and spleen. The formation of MHC II- peptide complexes on APC from thymus remained limited, similar to that conferred by peptide alone. The enhancement factor conferred by incorporation of peptide within the IgG was unexpectedly high (approximately 2-3 orders of magnitude), indicating that other factors, in addition to cellular handling (e.g. the above described pharmacokinetics and protective effects), were involved. Even 100 fold dose escalation of peptide alone, in saline or IFA, could not restore the *in vivo* loading of APC noted with peptide within IgG backbone.

Example 7 shows that among the three major APC subsets (DC, monocytes/macrophages and B cells) that express FcγR, the CD11c⁺ (DC) and CD11b⁺ (mostly monocytes) rather than B cells are the most potent on a per cell basis in presenting the peptide epitope subsequent to *in vivo* delivery via IgG backbone. The efficiency of APC loading and resulting presentation is substantially higher than that resulting from delivery of free peptide.

In vivo formation of MHC - peptide complexes on APC has been assessed subsequent to the administration of peptide epitope within IgG backbone followed by separation of various subsets of APC.

(A) Separation by using magnetic beads coupled with anti-MHC II or anti-CD11c mAb is carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as follows: spleens were processed to single cell suspension, red blood cells lysed, then cells washed, counted and resuspended in MACS buffer (PBS supplemented

with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative fraction runs through. After removal of the column from the magnetic field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in HL1 complete media and incubated in ELISPOT plates. Usually, from the total number of approximately 90 million splenocytes separated / 1 BALB/c mouse approximately 20 millions bind to magnetic beads coupled to anti-MHC II antibody and 3 millions interact with anti-CD11c mAb. Thus, less than 20 percent of splenocytes are able to present MHC class II restricted epitopes and approximately 2-3 percent are dendritic cells (see Figure 8A). These figures were confirmed by FACS analysis using specific antibodies.

(B) The *in vivo* loading of APC and formation of MHC II - peptide complexes on MHC II+ splenocytes has been assessed comparatively in Balb/c mice injected intravenously with 0.72 uM of recHA (I-Ed)-IgG ("IgHA") or 18 uM of HA peptide. At 24 hours, MHC class II+ APC were isolated from spleen by MACS as above, and incubated with peptide specific TcH (1×10^4 /well), in dose response manner. The next day the plate was centrifuged for 15min/4°C/1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4°C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 µl/well, centrifuging the plate for 3min/4°C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37°C with 200µl of the X-gal substrate freshly prepared as follows: 200µl of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope.

The results are expressed in Figure 8B as number of activated TcH / well. As a control, MHC II+ APC from naive BALB/c mice were incubated *in vitro*, overnight, with an optimal concentration of HA peptide (50ug/ml), extensively washed and incubated in different numbers with TcH as above. The results show that the formation of MHC II-

peptide complexes on splenic APC is at least 2 orders of magnitude more effective when the epitope is delivered within IgG backbone.

(C) A comparative assessment of the *in vivo* loading of various APC subsets after administration of recHA (I-Ed)-IgG has been carried out by magnetic separation of CD11c+, CD11b+ and CD19+ APC using the same protocol as above, using CD11c, CD11b and CD19 microbeads from Miltenyi Biotec. At 24 hours after intravenous injection with 0.72 μ M of recombinant immunoglobulin, the APC were isolated and incubated in a dose effect manner with a constant number of peptide specific TcH. After additional 24 hours, the assay was developed as above and results expressed as number of activated TcH / well. The results in Figure 8C show that on a per cell basis, use of peptide within IgG backbone led to predominant formation of immunogenic MHC II - peptide complexes on CD11c+ APC (dendritic cells), followed by CD11b+ monocytes and very ineffectively on CD19+ B cells.

(D) A comparison between the efficiency of *in vivo* formation of MHC II - peptide complexes on CD11c+ APC subsequent to peptide versus recombinant Ig delivery has been carried out following treatment of mice as described in the section B above. The CD11c+ splenic DC were isolated by MACS using CD 11c microbeads and incubated in different numbers with 1×10^4 TcH / well. Activated TcH were quantified as above and the results expressed as number of X-gal+ T cells / well. As a control, CD11c+ APC from naive mice loaded *ex vivo* with peptide were used as described in section B. The results in Figure 8D show that formation of MHC II peptide complexes was at least three orders of magnitude more effective when the peptide epitope was delivered within IgG backbone.

In conclusion, delivery of a peptide epitope within an IgG backbone resulted in more effective formation of MHC II - peptide complexes on CD11c+ DC. In addition, the efficiency of APC loading and formation of MHC II - peptide complexes was substantially higher when the peptide was delivered within IgG backbone. The results in Figs. 8A - 8D show that use of Fc γ R mediated delivery of peptides results in preferential formation of immunogenic MHC II - peptide complexes on CD11c+ and CD11b+ APC.

Example 8 shows a prolonged persistence *in vivo* of MHC-peptide complexes on APC (DC and monocytes) following administration via an IgG backbone.

The persistence of MHC II - peptide complexes on specific APC subsets was measured by magnetic separation of CD11c+ DC and CD11b+ monocytes at various intervals subsequent to intravenous injection of 2uM of recHA (I-Ed)-IgG. In brief, magnetic separation was carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as follows: spleens were processed to single cell suspension, red blood cells lysed, then cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative fraction runs through. After removal of the column from the magnetic field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in HL1 complete media and incubated. Different numbers of separated APC (A - CD11b+ monocytes, B - CD11c+ dendritic cells, C - whole splenocyte population) were incubated overnight with 1×10^4 TcH specific for the HA peptide.

As a control, APC from naive mice were used that were *in vitro* loaded with optimal amounts of HA peptide (50 μ g /ml), overnight and washed prior to incubation ("ctrl"). The next day the plate was centrifuged for 15min/4°C/1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4°C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 μ l /well, centrifuging the plate for 3min/4°C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37°C with 200 μ l/well of the X-gal substrate freshly prepared as follows: 200 μ l of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope and the number of activated TcH / well was plotted against the number of APC harvested at various intervals after treatment.

The results show long lasting expression of peptide onto endogenous MHC II, on both DC and monocytes. The complexes persisted between 1 and 2 weeks on these two APC subsets, in the conditions employed in this assay (strategy of APC separation and detection of MHC II - peptides).

Thus, the results in Figs. 9A - 9C show that the MHC-peptide complexes on selected APC formed subsequent to *in vivo* delivery of epitope via Ig are long-lived.

Example 9 shows that the γ chain of the Fc receptors (I and III) is essential for effective *in vivo* loading and presentation of a T cell epitope delivered within IgG backbone, by DC and monocytes.

The dependency of APC loading on the interaction with Fc γ R was studied by administration of 2 μ M of recHA(I-Ed)-IgG to BALB/c mice that lack a functional FcR gamma gene. One day after intravenous treatment, the CD11c+ and CD11b+ APC from spleen were separated by MACS. Separation by using magnetic beads coupled with anti-CD11c and anti-CD11b antibodies was carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as follows: spleens were processed to single cell suspension, red blood cells lysed, then cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative fraction runs through. After removal of the column from the magnetic field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in HL1 complete media and they were incubated in different numbers with 1×10^4 TcH specific for the HA peptide, overnight. As a control, APC from FcR gamma competent BALB/c mice were used. The next day the plate was centrifuged for 15min/4°C/1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4°C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 μ l /well, centrifuging the plate for 3min/4°C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37°C with 200 μ l/well of the X-gal substrate freshly prepared as follows:

200µl of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS).

The blue activated TcH were scored visually using the microscope.

The results are expressed as number of activated TcH / well for different APC subsets:

- 5 CD11c+ DC (A) and CD11b+ monocytes (B), or as control, whole splenic population (C).

The results (Fig. 10) clearly show that the formation of MHC II - peptide complexes on DC and monocytes, subsequent to IgG mediated delivery of peptide epitope, is critically dependent on ITAM+ FcγR that encompass the gamma chain. In
10 addition, gamma chain negative FcR isoforms cannot compensate for the absence of gamma chain+ FcR isoforms, in that regard.

Example 10 shows that the efficiency of T cell activation by a peptide delivered within the IgG backbone is dependent on the expression of γ chain+ FcγR (that promote activity) and FcγRIIB (that limit the activity) on APC. In addition, this experiment shows that ITIM-bearing FcγRIIB keeps in check the immune response to a peptide delivered within IgG backbone.

20 The differential role of FcR gamma+ versus gamma- isoforms to the immune response triggered by peptide epitope within IgG backbone, was studied by *ex vivo* loading of APC followed by adoptive transfer. Splenocytes from wild type, FcR gamma- or FcRIIB- BALB/c mice were incubated for 3 hours at 37°C as follows: 10 million cells / 1 ml of serum free HL-1 medium were admixed with 50µg/ml of HA 110-120
25 peptide or 10µg/ml of recHA(I-Ed)-IgG. Subsequently, the cells were washed and adoptively transferred into naive BALB/c mice (1 million cells suspended in 200µl serum free HL-1 and divided into 2 equal inoculi administered subcutaneously and intraperitoneally). After 2 weeks, the recipient mice were sacrificed, spleens harvested and the T cell response to the HA 110-120 peptide measured by ELISPOT analysis as
30 follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4µg/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4°C overnight. The next day, the

plates were washed 2 times with DMEM media and blocked with 200 μ l/well of DMEM complete containing FBS, for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 50 μ g /ml HA 110-120 peptide or just with media, to assess the
5 background.

Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer), and incubated with 100 μ l /well of biotinylated anti-cytokine Abs, 2 μ g /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C.

10 The next day plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. Plates were then allowed to dry at room temperature for 24 hours. The data were acquired using an automated system (Navitar,
15 Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results are expressed in Figure 11 as frequency of cytokine producing (A: IL-2, B: IL-4, and C: IFN-gamma) spot forming colonies obtained by incubation with medium only, or medium supplemented with HA 110-120 peptide (10 μ g/ml) (mean + SEM of triplicates, corresponding to 3 mice / group).

20 The results (Fig. 11) show that the expression of the gamma chain of ITAM+ Fc γ R isoforms is necessary for the induction of T cell response to APC loaded with peptide within IgG backbone. This was not necessary for the immunogenic effect of APC pulsed with peptide. Conversely, absence of ITIM+ Fc γ RII results in profound increase of the T cell response to APC pulsed with recombinant IgG but not HA peptide.

25 Together, these data show that the T cell response to recombinant IgG bearing a peptide epitope is determined by a complex interplay between ITAM+ and ITIM+ Fc γ gamma receptors on APC.

30 **Example 11 shows that unexpectedly, various subsets of APC *in vivo* loaded with epitope inserted within IgG backbone, differentially induce distinct regulatory subsets: while monocytes induce Th2 and Tr1 cells more effectively, both dendritic cells and monocytes induce Th3 cells. In addition, on a cell population level, the**

CD11b⁺ monocytes are more potent than the dendritic cells in triggering a regulatory response following IgG-mediated delivery of T cell epitope.

Four BALB/c mice were injected intravenously with 2 μ M of recHA (I-Ed)-IgG.

5 One day later, the spleens were harvested and APC were isolated by MACS using anti-CD11c, anti-CD11b or anti-CD19 monoclonal antibodies coupled with magnetic beads. Separation by using magnetic beads coupled with anti-CD11b, anti-CD11c and anti-CD19 mAb is carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as follows: spleens were processed to single cell suspension, red blood

10 cells lysed, then cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative fraction runs through. After removal of the column from the magnetic field, the

15 magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in serum free HL-1 medium as follows: 3x10⁶/ml CD11c⁺ DC, 28x10⁶/ml CD11b⁺ or 84x10⁶/ml of CD19⁺ B cells. This numerical distribution respects the proportion of the APC subsets isolated from the splenic tissue. Cells were transferred into naïve BALB/c mice by subcutaneous and intraperitoneal injection (100+100 μ l /

20 mouse, n=2 mice / group). At 2 weeks after the adoptive transfer, mice were sacrificed and T cell response measured by ELISPOT (IL-4 and IFN- γ) or measurement of cytokine production in cell culture supernatants, by ELISA TGF- β 1 kit (R&D Systems, cat # DY240) and IL-10 kit (Biosource international, cat#KMC0104).

The results are expressed in Figure 12 as number of spot forming colonies / spleen

25 (average of duplicates; panels A, B) or amount of cytokine measured in supernatants (pg/ml, average of duplicates; panels C, D) at various concentrations of HA peptide used for restimulation.

The results (Fig. 12, panels A - D) clearly show that unexpectedly, and in contrast with the potency / cell basis (Example 8), at the organism level, the CD11b⁺ monocytes

30 have the highest impact on the immune response to a peptide epitope delivered within the IgG backbone. Thus, the CD11b⁺ APC subset induced both Th2, Tr1 and Th3 cells. In contrast, the CD11c⁺ DC induced Th3 cells and more reduced Th2 response. Finally,

despite their substantial number, the CD19⁺ B cells were poor inducers of T cell immunity to the peptide epitope within the IgG backbone. No significant Th1 responses were induced by either of the APC subsets tested.

5 Example 12 shows that the loading of APC *in vivo* with a peptide delivered within IgG backbone results in induction of Th2 but not Th1 immunity.

BALB/c mice were immunized with 100 µg of recHA (I-E_d)-IgG ("IgHA"), or a molar equivalent amount of HA peptide epitope (2µg), by subcutaneous injection and sacrificed 2 weeks later. The immune response was measured by ELISPOT analysis using splenocytes from treated mice as responders, and mitomycin-treated splenocytes from naïve mice as stimulators, as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 20 µg /ml HA 110-120 peptide or just with media, to assess the background.

Stimulator cells were prepared from naïve mice as follows: single cell suspension was prepared from spleens, red blood cells were lysed, cells were washed, resuspended in HL1 complete and mitomycin treated for 30 minutes. Afterwards, cells were washed 3 times, counted and resuspended in serum free HL1 media. The plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer), and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C.

The next day, the plates were washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours. The data were acquired using an automated system

(Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD).

The results are expressed in Figure 13 as number of IL-4-producing (A) or IFN- γ producing (B) T cell colonies / spleen (mean \pm SEM of triplicates) when splenocytes were restimulated with 10 μ g/ml of HA peptide or cell culture medium alone. Thus, this Example shows that Fc γ R-mediated delivery of T cell epitope within recombinant Ig backbone results in Th2 rather than Th1 response.

Example 13 shows that the repeated loading of APC *in vivo* with a peptide delivered within IgG backbone results in induction of Th3 and Tr1 immunity.

BALB/c mice were immunized with 40 μ g of heat aggregated (15 mins at 63 $^{\circ}$ C) of recHA (I-Ed)-IgG ("IgHA") administered by intranasal instillation boosted 2 weeks later by subcutaneous injection with 100 μ g of recombinant immunoglobulin in saline. As controls, mice primed with heat aggregated IgG2b isotype control were used. After an additional 2 weeks, the mice were sacrificed and T cell response assessed by in vitro restimulation of splenocytes with HA peptide by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4 μ g/ml for anti-IL2 and anti-IL4, and 8 μ g/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μ l/well) at 4 $^{\circ}$ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200 μ l/well of DMEM complete containing FBS, for an hour at 37 $^{\circ}$ C.

Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 20 μ g /ml HA 110-120 peptide or just with media, to assess the background. Plates were incubated 72 hours at 37 $^{\circ}$ C, 5% CO₂. After 3 days, plates were washed 5 times with PBS-tween20 0.05% (washing buffer), and incubated with 100 μ l /well of biotinylated anti-cytokine Abs, 2 μ g /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4 $^{\circ}$ C.

The next day, plates were washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped

by washing the plate twice with tap water. Plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The TGF-beta and IL-10 production were measured by ELISA TGF- β 1 kit (R&D Systems, cat # DY240) and IL-10 kit (Biosource international, cat#KMC0104). The results are expressed as cytokine concentration (average of triplicates) after subtraction of background.

The data, as shown in Figure 14, show that mucosal priming with epitope bearing recombinant immunoglobulin resulted in differentiation of Th3 and Tr1 cells that were expanded subsequently by systemic boosting.

Example 14 shows that only a virus, but not the conventional adjuvant CFA, was able to trigger significant Th1 response to a peptide epitope inserted within the IgG backbone.

BALB/c mice were immunized intraperitoneally with 100ug of recHA (I-Ed)-IgG in saline, emulsified in Complete Freund's Adjuvant ("CFA") or with 105 TCID₅₀ of influenza virus strain WSN, that bears the HA epitope. At 2 weeks after immunization, the mice (n=3/group) were sacrificed and the T cell response to HA peptide measured by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 μ g/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μ l/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200 μ l/well of DMEM complete containing FBS, for an hour at 37⁰ C.

Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 20 μ g /ml HA 110-120 peptide or just with media, to assess the background.

Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer), and incubated with 100 μ l /well of biotinylated anti-cytokine Abs, 2 μ g /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C. The next day, plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in

ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results are represented as mean \pm SEM of frequency of cytokine producing colonies in the spleen.

The results in Fig. 15 show that a peptide epitope within the IgG backbone triggers a cellular response of Th2 profile that is enhanced but not switched by a conventional adjuvant (CFA). In contrast, the profile afforded by live virus immunization was Th1 biased.

Example 15 shows that the presentation of peptide epitope subsequent to IgG mediated delivery results in a T cell response that could be further manipulated by increasing co-stimulation with anti-CD40mAb, recombinant IL-12 or synthetic dsRNA.

Dendritic cells from naive BALB/c mice were harvested by MACS from splenic cell suspensions as follows: Separation by using magnetic beads coupled with anti-CD11c was carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as follows: spleens were processed to single cell suspension, red blood cells lysed, the cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative fraction runs through. After removal of the column from the magnetic field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in HL1 complete media and were pulsed *ex vivo* in serum free HL-1 medium for 2 hours, at a concentration of 3 million / ml, with 50ug/ml of recHA(I-Ed)-IgG alone or supplemented with 5ng/ml of recIL-12, 50ug/ml of double stranded RNAs (pA:pU or pI:pC). Alternatively, the cells were incubated with recombinant Ig and wells precoated with 10ug/ml of anti-CD40 mAb. The cells were harvested, washed and

adoptively transferred to naive BALB/c mice (300,000 delivered half subcutaneously and half intraperitoneally) in serum free HL-1 medium.

At 2 weeks, the mice were sacrificed and T cell responses measured against HA by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C.

Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 50 µg /ml HA 110-120 peptide or just with media, to assess the background. Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C. The next day plates were washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results are shown as mean + SEM (n=3) of the frequency of spot forming colonies associated with IL-2 or IL-4 production, after subtraction of the background, for each *ex vivo* stimulatory combination.

The results in Fig. 16 show that peptide presentation by APC, subsequent to loading with antigen by using recombinant IgG as delivery platform, occurs in context of limited co-stimulation. IL-12, anti-CD40 or synthetic dsRNA can all enable APC loaded with antigen via FcγR, to prime IL-2 and enhanced IL-4 producing T cell immunity against the cognate (HA) peptide.

Example 16: The activity of the long-lived IL-4 producing Th2 cells triggered by *in vivo* loading of APC with IgG-peptide is dependent on the continuous interaction with endogenous APC and requires competent CD4.

5 BALB/c mice were immunized with 100 ug of recHA (I-E_d)-IgG or HA peptide subcutaneously, sacrificed at 2 weeks and the T cell response measured by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml anti-IL4, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plate was washed 2 times with DMEM
10 media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C.

Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 20 µg /ml HA 110-120 peptide or just with media, to assess the background. The plate was incubated 72 hours at
15 37⁰ C, 5% CO₂. After 3 days, the plate was washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C.

The next day, the plate was washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was
20 developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plate was then allowed to dry at room temperature for 24 hours. The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD).

(A) During the HA stimulation phase, blocking anti-CD4 or anti-CD8 mAb was added
25 at 10ug/ml in selected wells. The results are expressed in Fig. 17A as mean+SEM of number of HA-stimulated IL-4 producing colonies per spleen, after subtraction of background (n=3 mice / group).

(B) Splenocytes from mice immunized with recombinant Ig as above, were incubated in elispot plate as is or after magnetic depletion of endogenous MHC II+ APC with
30 MHC II+ from naive BALB/c mice, with medium alone or in the presence of 10ug / ml of HA peptide. Separation by using magnetic beads coupled with anti-MHC II was carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as

follows: spleens were processed to single cell suspension, red blood cells lysed, then cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative fraction runs through. After removal of the column from the magnetic field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in HL1 complete media and were incubated in the ELISPOT assay, protocol to follow. The ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 50 µg /ml HA 110-120 peptide or just with media, to assess the background.

Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C.

The next day, plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. Plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD) and the results expressed as mean ± SEM of the frequency of IL-4 producing T cells. The results in Figs. 17A - 17B show that the activity of HA specific IL-4 producing T cells triggered by administration of recHA(I-Ed)-IgG is dependent on CD4 rather CD8. In addition, the

long lived IL-4 production by primed T cells depends on stable interaction with endogenous APC.

5 **Example 17 shows that FcγR-mediated delivery of a T cell epitope is more effective than the peptide in differentially affecting the phenotype of activated, specific T cells: dose-dependent down regulation of IL-2, IFN-γ, and IL-4, with up-regulation of IL-10 and TGF-β.**

Activated SFERFEIFPKE-specific T cells were separated from BALB/c mice
10 immunized 2 weeks previously with 100μg peptide in CFA. They were incubated with mitomycin treated splenocytes in the presence of various amounts of recHA(I-Ed)-IgG or corresponding peptide. The expansion and cytokine production (IFN-γ, IL-4, IL-2) was estimated by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-
15 IL4, and 8 μg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200μl/well of DMEM complete containing FBS, for an hour, at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 20 μg /ml HA 110-120
20 peptide or just with media, to assess the background.

The plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 μl /well of biotinylated anti-cytokine Abs, 2 μg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C. The next day, the plates were washed five
25 times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with
30 ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). In addition, TGF-β and IL-10 production were measured by ELISA at 48 hours after incubation using TGF-β1 kit (R&D Systems, cat # DY240) and IL-10 kit (Biosource international,

cat#KMC0104). The results are expressed as frequency of spot forming cells (SFC) or concentration of cytokine versus amount of antigen added *in vitro*.

The results in Fig. 18 show that the IgG mediated delivery of a T cell epitope has a profound and differential effect on the expansion and cytokine production by activated T cells: IL-2, IFN- γ and surprisingly IL-4, were down-regulated in a dose-related manner. The Ig-peptide was substantially more effective in modulating the cytokine production, as compared to the peptide itself. In contrast, only the Ig-peptide turned on effectively the production of IL-10 and TGF-beta in a dose-dependent manner. Thus, the T cell epitope in context of Ig backbone, but not separately, differentially modulated the function of activated cells.

Example 18 shows that surprisingly, a peptide delivered within the IgG backbone, that is not an immune complex nor is a receptor cross-linking antibody, results in induction of a class I restricted immune response. This response had a different profile from that triggered by live virus (Tc2 type consisting in IL-4 but not IFN- γ production).

BALB/c mice were injected with 50 μ g of recNP(Kd)-IgG encompassing the MHC class I-restricted peptide TYTQTRALV (Seq. I.D. No. 6) by subcutaneous injection. The mice were sacrificed 2 weeks later and peptide-specific cytokine production was measured by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4 μ g/ml for anti-IL2 and anti-IL4, and 8 μ g/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μ l/well) at 4 $^{\circ}$ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200 μ l/well of DMEM complete containing FBS, for an hour at 37 $^{\circ}$ C.

Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with various concentrations of NP peptide. Plates were incubated 72 hours at 37 $^{\circ}$ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 μ l /well of biotinylated anti-cytokine Abs, 2 μ g /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4 $^{\circ}$ C. The next day the plates were washed five

times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

5 The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results are expressed in Figure 19A as total number of spot forming colonies (SFC) / spleen (mean of n=3). As controls, naïve mice or mice injected intraperitoneally with 10^5 TCID₅₀ of live WSN influenza virus were used.

10 The results in Fig. 19A - 19B show that in contrast to viral immunization with an influenza virus strain bearing the cognate peptide, Ig-mediated peptide delivery was ineffective in triggering IFN- γ producing Tc1 cells. However, Ig-peptide administration still resulted in formation of MHC class I-peptide complexes and induced significant NP-specific MHC class I-restricted T cell immunity consisting in IL-4 producing Tc2 cells.

15 **Example 19 shows that *in vivo* loading of selected APC with disease associated epitopes suppressed an aggravated form of autoimmunity by expanding rather than ablating, epitope-specific autoreactive T.**

20 SJL mice were injected subcutaneously with 200 μ l of rat brain homogenate emulsified in Complete Freund's Adjuvant and boosted with 50ng of pertussis toxin at 6 hours and 2 days. The mice developed an aggravated, progressive form of paralytic disease. Half of the mice received via subcutaneous injection a combination of recombinant immunoglobulins bearing the MBP and the PLP epitopes (recMBP(I-As)-
25 IgG; recPLP(I-As)-IgG), respectively (150 μ g/molecule, on day 8, 12, 18 after induction of disease). In panel A, the mean clinical score for treated and non-treated mice is represented, respectively (n=8).

 After a period of observation of 70 days, the mice were sacrificed, spleens harvested and elispot analysis carried out as follows: the ELISPOT plates (Millipore,
30 Molsheim, France) were incubated with purified anti-cytokine Abs (4 μ g/ml for anti-IL4, and 8 μ g/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μ l/well)

at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 1x 10⁶ /well together with 20 µg /ml of peptides (PLP or MBP) or just with media, to assess the background.

Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1% (ELISPOT buffer) overnight at 4⁰ C. The next day, the plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. Plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results (Figure 20 B) were expressed as frequency of IL-4 producing T cell colonies in the absence of added PLP peptide plotted against the frequency of IFN-γ-producing T cells in condition of peptide stimulation. Mice progressing to full-blown limb paralysis (score equal to or higher than 1.5) were represented with closed symbols. Mice that did not progress to limb paralysis were represented with open symbols. In Figure 20C, the total number of IL-4 spot forming colonies / spleen (mean±SEM) in condition of *in vitro* stimulation was represented with nil, MBP or PLP peptide. An additional control, consisting of splenocytes from mice treated with IgG2b isotype control, has been included. In parallel, *in vitro* culture was carried out in the presence of neutralizing anti-IL-4 mAb (40µg/ml) and the number of IFN-γ-producing T cells was represented in the panel D.

The results in Figs. 20A - D show that co-administration of MBP and PLP epitopes by using recombinant IgG significantly curbed the chronic progression of disease. The mice protected from paralysis developed unexpectedly, an enhanced reactivity to self-epitopes MBP and PLP, manifested by increased basal and peptide-stimulated IL-4 or IFN-γ production, respectively. Finally, the reactivity of IFN-γ-

producing T cells is kept in check by IL-4 suggesting a complex immunomodulatory mechanism triggered by IgG-mediated delivery of epitopes.

Example 20 summarizes the impact of IgG / FcγR-mediated delivery of epitopes on the T cell response, based on data provided in the Examples 1-19.

First, the loading of APC T cell response to IgG-mediated delivery of T cell epitopes is controlled by two functionally opposing receptors: ITIM and ITAM Fc (gamma⁺)-bearing receptors on APC. ITIM⁺ FcγRIIB limits the degree of activation of T cells and gamma⁺ FcRs are required for effective formation of MHC-peptide complexes when epitopes are delivered via the IgG backbone. Such *in vivo* delivery of epitope results in effective formation of MHC – peptide complexes on peripheral CD11c⁺ and CD11b⁺ APC, but not thymic APC. However, the interplay between ITIM⁺ and ITAM⁺ FcγRs makes the nature and magnitude of resulting T cell response difficult to predict without experimentation.

The data in Fig. 21 show that IgG-delivery of peptide epitope results in exposure of T cells to peptide-loaded APC in context of limited co-stimulation, having a differential effect on naïve versus activated T cells: 1) de novo induction of Th2, Tc2, Th3, Tr1 cells and, 2) downregulation of activated Th1, Th2 cells with stimulation of activated Tr1 and Th3 cells. The overall effect is immunomodulatory, rather than pro-inflammatory (associated with Th1 and Tc1 immunity).

Example 21. Naturally occurring dsRNA bridges the innate with adaptive immune response. Example 21 shows that natural, non-infectious double stranded RNA produced during infection with influenza virus, has substantial effects on the specific immune response to a protein antigen.

Permissive MDCK cells were infected with WSN influenza virus (10⁸ TCID₅₀ / 1x10⁹ cells) and after 24 hours, the cells were harvested, washed and the total RNA extracted using an RNA separation kit (Qiagen, Valencia, CA). The RNA was further purified by treatment with RNase-free DNaseI (Stratagene, San Diego, CA). The single stranded RNA in the samples was then removed by 30 minutes incubation at 37°C with 5U of S1 nuclease (Ambion, Inc., Austin-TX) / μg of RNA. The RNA was analyzed prior

to and subsequent to the digestion by gel electrophoresis. The absence of infectious properties of the purified dsRNA was confirmed by standard influenza virus titration. As a control, material purified and treated similarly, from 10^9 non-infected MDCK cells was used. The concentration of nucleic acid was measured by spectrophotometry (A_{260nm}) and the absence of endotoxin confirmed by Limulus assay. The purified dsRNA and control RNA were used individually, or as a mixture with gp140 recombinant antigen (25 μ g of RNA and 2 μ g of antigen in 25ml of sterile PBS).

After demonstrating lack of infectivity, 40 μ g of dsRNA or control RNA were admixed with 40 μ g of recombinant truncated antigen (gp140 of HIV envelope) and were administered to BALB/c mice by intranasal instillation (n=3/group). Additional controls were animals immunized with 40 μ g of gp140 protein in saline (n=3 / group). The mice were boosted once, at 2 weeks after priming. Blood was harvested 2 weeks after the boost, sera prepared and the antibody response against gp140 measured by ELISA. In brief, wells were coated with antigen (2 μ g/ml of gp140) and blocked with SeaBlock (Pierce, Rockford-IL, catalog # 37527). Serial dilutions of serum and bronchoalveolar lavage fluid were incubated for at least 2 hours at room temperature. After washing, the assay was developed with anti-mouse IgG antibody coupled with alkaline phosphatase (Sigma, cat# A7434) followed by addition of substrate (pNPP, Sigma, cat# N2765) and measurement by using an automatic microtiter plate reader (Molecular Devices, ThermoMax) equipped with SoftMax software.

In Fig. 22A, the general principle of the experiment is illustrated. In Fig. 22B, the absorption after assay development is represented, corresponding to various serum dilutions, in case of whole IgG. In Fig. 22B, the absorption at 1/50 serum dilution, in case of IgG2a and IgG1 antibody isotypes, is represented.

Overall, the data in Figs. 22A - B show that natural, non-infectious dsRNA from influenza virus-infected MDCK cells, has an unexpected enhancing effect on the adaptive response to a prototype antigen. Both IgG1 and IgG2a antibody responses were increased showing that a strong T helper1 and T helper 2 response was induced.

Example 22. Effects of selected RNA motifs on the innate immune response: heterogeneous motifs. This Example shows, unexpectedly, that different synthetic

RNA motifs have a distinct effect on the adaptive specific immune response to a protein antigen.

Figure 23A shows an extensive library of synthetic RNA motifs, that was grouped in pools and used for a two-tier screening process as follows:

(A) The mice were immunized intratracheally with RNA pools, followed by 2 boosts two weeks apart, carried out by intranasal instillation. The antibody response measured (Fig. 23 B) by ELISA was expressed as mean \pm SEM of IgG endpoint titers (n=4/group). As controls, dose-matched OVA in sterile PBS was used, OVA with cholera toxin subunit B (CTB) and PBS alone, respectively. In brief, wells were coated with antigen (10 μ g/ml of OVA) and blocked with SeaBlock (Pierce, Rockford-IL, catalog # 37527). Serial dilutions of serum and bronchoalveolar lavage fluid were incubated for at least 2 hours at room temperature. After washing, the assay was developed with anti-mouse IgG antibody coupled with alkaline phosphatase (Sigma, cat# A7434) followed by addition of substrate (pNPP, Sigma, cat# N2765) and measurement by using an automatic microtiter plate reader (Molecular Devices, ThermoMax) equipped with SoftMax software.

(B) The effect of various dsRNA motifs on the induction of antibody response to OVA: the results are expressed as in Fig. 23 C. The data are representative for two independent experiments. INSET: the ratio between mean IgG2a and IgG1 titers to OVA. For this purpose, biotin-conjugated anti-mouse IgG1 and IgG2a antibodies were used followed by incubation with streptavidin-AKP conjugate. The order from left to right is similar as in the main panel in Fig. 23C: PBS OVA, CTB OVA, pC:pG OVA, pI:pC OVA and pA:pU OVA.

(C) The magnitude and profile of T cell response induced by OVA together with various dsRNA motifs, in female C57BL/6 mice. For the measurement of cellular response, splenic cell suspensions were obtained by passing the organ through 70 micron nylon Falcon strainers (Becton Dickinson, cat# 352350) followed by lysis of red blood cells with red blood cell lysis buffer (Sigma, cat# R7757). The lymphocytes from the pulmonary associated lymphoid tissue were isolated by collagenase (Sigma, cat# C9891) digestion of lung tissue followed by Ficoll-Paque (Amersham Pharmacia, cat# 17-1440-

02) gradient centrifugation. The T cell response was measured by ELISPOT analysis as follows: 96-well 45 micron mixed cellulose ester plates (Millipore, cat#MAHA S4510) were coated with 4µg/ml of rat anti-mouse anti-IFN γ , IL-2 or IL-4 monoclonal antibodies (BD-PharMingen, cat#554430, cat#18161D, cat# 554387 respectively). After blocking with 10% FCS in sterile saline for 1 hour at 37°C, spleen cell suspensions were added at 5x10⁵ cells / well, with or without antigens / peptides. For stimulation, graded amounts of antigen (OVA) were used. At 72 hours after stimulation, the assay was developed with biotinylated rat anti-mouse cytokine antibodies (BD-PharMingen) followed by streptavidin-HRP (BioSource Int., Camarillo, CA) and insoluble AEC substrate. The results were measured using an automatic imaging system (Navitar/Micromate) equipped with multiparametric-analysis software (Image Pro, Media Cybernetics). The results are expressed in Figure 23 D as mean \pm SEM of the number of IFN- γ and IL-4 spot-forming-colonies (SFC) per spleen (n=4/group). The results are representative for two independent experiments.

The results in Figs. 23B - D show that different synthetic RNAs have an enhancing effect on the B and T cell response to a prototype protein antigen. In addition, different motifs, comprising specific nucleotide combinations, have specific effects in terms of T1 versus T2 induction and subsequently, immunoglobulin isotype switching.

Example 23. Use of selected synthetic RNA motifs facilitates the induction of MHC class I-restricted Tc1 cells, producing IFN- γ .

(A) Cross-priming stimulated by dsRNA motifs was studied in BALB/c mice treated (priming plus 2 boosts) with 10µg of recombinant-engineered HIV gp140 antigen together with pA:pU. The response was measured by ELISPOT analysis as described in Example 22, using *in vitro* stimulation with the MHC class I-restricted cognate peptide R10K derived from the V3 domain. As a control, dose-matched gp140 antigen was used. The results are expressed in Figure 24A as mean \pm SEM of the number of IFN- γ and IL-4 SFC / spleen (n=4/group).

(B) Cross-priming stimulated by dsRNA motifs was studied in C57BL/6 mice treated with 100µg of whole OVA together with pA:pU by ELISPOT analysis as described in

Example 22, using *in vitro* stimulation with the MHC class I-restricted peptide SIINFEKL (Seq. ID No.). As a control, dose-matched OVA antigen in saline or sterile PBS was used. The results are expressed in Figure 24B as mean \pm SEM of the number of IFN- γ and IL-4 SFC / spleen (n=4/group).

5 The results in Figures 24A - B show that a selected synthetic RNA motif was able to promote increased T cell immunity to different MHC class I-restricted peptides encompassed within larger antigens (polypeptides). This immune response comprised a Tc1 component, consisting in IFN- γ -producing MHC class I-restricted T cells.

10 **Example 24 shows that unexpectedly, different synthetic RNA motifs bind to different receptors; in other words, there are multiple receptors that discriminate among RNA motifs.**

In vitro binding of CD11b⁺ APC by fluorescently-tagged pA:pU was measured by
15 FACS analysis. The MACS-separated APC were incubated at 4°C for 30 minutes with 10 μ g/ml of tagged pA:pU ([pA:pU]-F), washed and analyzed. Alternatively, APC were preincubated for 10 minutes with 20 or 100 μ g/ml of non-tagged pA:pU, pA or pI:pC respectively, before staining with tagged pA:pU and FACS analysis. The profiles of stained (open area), non-stained (filled area) cells and the percentage of highly stained
20 APC were represented in each panel, with logarithmic x axis. The data are representative of two independent measurements with 10,000 events acquired for each sample.

Materials:

1. Mouse CD11b, CD11c Magnetic Separation Beads: Miltenyi Biotec, cat#130-049-
25 601, cat#130-052-001 respectively;
2. ULYSIS Nucleic Acid Labeling Kit: Alexa 488, Molecular Probes cat#U21650;
3. RNA Motifs:
 - pA:pU, (Sigma, Lot #22K4068);
 - pI:pC, (Sigma, Lot# 52K4047);
 - 30 • pA, (Sigma, Lot#22K4022);
4. FACS Buffer: PBS, 1% FCS, 0.1% sodium azide;
5. MACs buffer: PBS, 2mM EDTA, 0.5% BSA;

6. Collagenase Buffer: 0.225mg BSA, 0.0062mg collagenase in 50ml RPMI; and,
7. 70um cell strainer: (Falcon / Becton Dickinson, cat#352350.

Methods:

5 I. Labeling of RNA Motifs:

1. In the following protocol, each RNA motif was tagged with the ULYSIS Alexa 488 label.

II. Splenocyte preparation:

- 10 1. Isolate splenocytes and lung cells from 4 female C57 BL/6 mice;
 - Lung cells, in contrast to splenocytes, must be minced and incubated in collagenase buffer for 30 minutes at 37°C prior to the following step;
 - Pass through 70um falcon cell strainer;
 - Wash and resuspend in MACS buffer:
- 15 2. Label with either CD11b or CD11c specific MACS beads following suggested protocol;
3. Cells were then treated with:
 - Non-tagged pA, pA:pU, or pI:pC (20 or 100ug/ml) for 10 minutes at room temperature;
 - 20 • ULYSIS tagged pA or pA:pU was added at 1.5ug/tube and 10ug/tube, respectively, to match dye:dsRNA ratio of each motif.
4. Mix and incubate 30 minutes on ice.
5. Wash once and resuspend in FACS buffer

25 III. Flow Cytometry:

Run flow cytometric analysis to determine / compare competitive inhibition of tagged versus non-tagged RNA motifs and cell receptor binding.

- The results in Figure 25 show that pA:pU and pI:pC bind to different cellular
- 30 receptors. Since pI:pC binds to TLR3, it results that additional receptors distinct from TLR3 are involved in RNA recognition immune function.

Example 25 shows that selected synthetic RNA motifs trigger *in vivo* expression of chemokine genes, of importance for immunological activity.

5 Local up-regulation of chemokine gene-expression by dsRNA motifs was measured by DNA array technique using RNA from the pulmonary tissue, extracted one day after the administration via the respiratory tract. Total RNA was isolated from lungs using an RNeasy kit (Qiagen, Valencia, CA). The RNAs were further purified by treatment with RNase-free DNase I (Stratagene, San Diego, CA). DNA array was
10 performed by using the Nonrad-GEArray kit from SuperArray Inc. (Bethesda, MD). Briefly, cDNA probes were synthesized using MMLV reverse transcriptase with dNTP mix containing biotin-16-dUTP. The GEArray membranes were prehybridized at 68°C for 1-2 hours. The hybridization was carried out by incubation of the membranes with biotin-labeled cDNA. The hybridized membranes were washed in 2xSSC - 1% SDS
15 twice and 0.1xSSC - 0.5% SDS twice. The membranes were further incubated with alkaline phosphatase-conjugated streptavidin (BioSource Int., Camarillo, CA) and finally developed with CDP-Star chemiluminescent substrate. The intensity of signal was measured with Image-Pro analysis system equipped with Gel-Pro software (Media Cybernetics, Silver Springs, MD).

20 The results are expressed as fold-increase of gene expression, over expression levels measured in the pulmonary tissue of non-treated mice. The pattern of chemokine expression triggered by dsRNAs (50 µg of pA:pU and pI:pC, respectively) was compared to that induced by 1 µg of LPS. The chemokines that selectively bind to receptors on Th1 and Th2 cells were indicated with continuous and interrupted contours, respectively.

25 The results in Figure 26 show that pA:pU and pI:pC trigger expression of a wide range of chemokines and that the expression pattern is motif-dependent and different from that elicited by LPS (endotoxin).

Example 26 shows that selected synthetic RNA motifs mobilize an immune defense that is capable to control infection with a pulmonary virus.

30 dsRNA motifs display differential ability to mobilize immune defense against influenza virus infection. C3H/HeJ mice were treated via the respiratory route with 50µg

of pI:pC, pA:pU or 50µl of saline one day before and after pulmonary infection with a sublethal dose of influenza virus. For virus challenge, C57BL/6 and TLR4-/- C3H/HeJ mice under Metofane anesthesia were infected with sublethal doses (10^4 tissue culture infective doses 50% - TCID₅₀) of live WSN virus, via the nasal route. On day 5 after infection, the mice were sacrificed, lungs retrieved, homogenized and stored at -70°C. The virus titers were measured by 48-hour incubation of serial dilutions of samples with permissive MDCK cells, followed by standard hemagglutination with chicken red blood cells (From Animal Technologies). The endpoint titers were estimated in triplicate measurements by interpolation and expressed as TCID₅₀ / organ (means \pm SEM; n=6/group; results are representative of two independent studies in C3H/HeJ TLR-4-/- and competent mice). Similar results were obtained in TLR4 competent, C57BL/6 mice.

Thus, the results depicted in Figure 27 show that the control of replication of influenza virus can be achieved by using selected synthetic RNA motifs (dsRNA1 is pA:pU and dsRNA2 is pI:pC).

Example 27 shows that co-administration of selected synthetic RNA motifs breaks tolerance to high dose standard antigen.

dsRNA motifs prevent high-zone tolerance in mice injected with human IgG. The mice (C57BL/6) were initially injected intravenously with a toleragenic dose of 200µg of hIgG alone (closed symbols) or together with 100µg of pI:pC or pA:pU (open symbols) and subsequently boosted subcutaneously with an immunogenic dose of 100µg of hIgG emulsified in CFA. The titer of antibodies against hIgG was measured by ELISA (as detailed in the Example 23, with the difference consisting in use of 10µg/ml of hIgG for coating) at various intervals after the first injection. As a control, mice immunized with 100µg of hIgG emulsified in CFA were included and represented the maximal titer on the graph (interrupted line).

The results are represented in Figure 28 as means \pm SEM of endpoint titers (n=5/group). Similar results were obtained in TLR4 deficient (C3H/HeJ) and LPS-responsive C3H/SnJ mice. Thus, the results in Figure 28 show that selected synthetic RNA motifs pI:pC and pA:pU largely prevent high zone tolerance that is usually associated with administration of large amounts of purified protein.

Example 28 shows that selected RNA motifs induce differential cytokine production by human APC.

5 Human THP-1 monocytic cells, following differentiation, were incubated with different concentrations of synthetic RNA (pA:pU, pI:pC or pA) for 24 hours, and the cell supernatants collected. The concentration of IL-12 and TNF- α were measured by ELISA. The results are expressed in Figure 29 as pg/ml (concentration) for each cytokine and culture condition.

10 **Materials:**

1. THP-1 Human monocytic cell line: ATCC, cat # TIB-202;
2. IL-12 Cytokine: Human ELISA, IL-12 ultra sensitive (US) cat# KHC0123;
3. TNF alpha Cytokine: Human ELISA, TNF alpha cat# KHC3012;

15 **4. RNA Motifs:**

- pA:pU, (Sigma, Lot #22K4068);
- pI:pC, (Sigma, Lot # 52K4047); and,
- pA, (Sigma, Lot #22K4022).

20 **Method:**

1. The THP-1 cells were allowed to differentiate following addition of 10ng/ml PMA in media containing 10% FCS.
2. After gently washing cells and adding non-FCS containing media (HL-1), treatments (RNA motifs and controls) were added at concentrations of from 3 to 100 μ g/ml on
25 top of adherent THP-1 cells.
3. After 24 hours incubation, cell supernatants were harvested and IL-12 and TNF alpha concentrations were measured by ELISA.

30 The results in Fig. 29 show selected synthetic RNA motifs effect on human monocytic cells; in addition, this effect is heterogeneous, depending on the chemical structure of the motifs (nucleotide composition). Selected but not all synthetic RNA

motifs are able to trigger IL-12 production, an important T1 regulatory cytokine, by human monocytic cells.

Example 29 shows that two distinct synthetic RNA motifs bind to human THP-1 monocytic cells in a manner demonstrating interaction with different receptors.

THP-1 cells were incubated at for 15 minutes at room temperature with different amounts of non-labeled synthetic RNA. Subsequently, tagged pA:pU was added for 30 minutes at 4⁰C, cells washed and the fluorescence quantified by FACS analysis.

The results are expressed in Figs. 30A - 30B as histograms corresponding to the large cell subset (A) and total cell population (B). Percentages of stained cells were represented on each Figure.

Materials:

1. ULYSIS: Nucleic acid fluorescent label (Molecular Probes, cat# U-21650).
2. RNA Motifs:
 - pA:pU, (Sigma, Lot #22K4068);
 - pI:pC, (Sigma, Lot # 52K4047);
3. Detoxi-Gel column: (Pierce, cat#20344).

Method:

Labeling of Polyadenylic-Polyuridylic Acid (pA:pU):

1. Following removal of endotoxin using a Detoxi-Gel column, pA:pU was labeled with the Alexa Fluor 488 fluorescent dye using the ULYSIS nucleic acid labeling system.

2. Briefly:

- The pA:pU was precipitated using sodium acetate and ethanol at -70°C;
- The pA:pU was heat denatured and labeled with the Alexa Fluor 488 reagent at 90°C; and,
- The reaction was stopped and the labeled pA:pU was ethanol precipitated.

Cell treatment:

1. THP-1 cells were suspended at 2X10⁶ cells /ml;

2. 50µl of above suspension (5×10^4 cells) were placed in 12X75 mm tubes;
3. Non-tagged pA:pU or pI:pC were added to the THP-1 cells at a concentration of either 20 or 100µg/ml and incubated 15 minutes;

ULYSIS labeled pA:pU was added at a concentration of 100 ug/ml for 30 minutes on ice.

- 5 4. The THP-1 cells were washed once and suspended in FACS buffer followed by flowcytometric analysis to determine relative fluorescent differences between different treatment populations.

The results in Figures 30A - 30B show that non-tagged pA:pU but not non-tagged pI:pC was able to compete out the binding of tagged pA:pU to human THP-1 monocytic cells, both at the level of large cell subset and whole population.

Example 30 shows how the adjuvant synthetic RNA should be prepared and purified prior to use in its most effective format.

15 The bulk synthetic RNA material is obtained by standard methods of organic synthesis. Afterwards, the material is dissolved in sterile endotoxin-free saline, passed through endotoxin removal columns until the concentration of LPS is below 0.005EU/µg. The measurement of LPS is carried out by standard Limulus assay. Subsequently, the material is fractionated by a series of centrifugation steps through filters of defined
20 porosity (see Fig. 31).

A useful fraction comprises synthetic RNA of less than 20 to maximum 100bp size, however, larger RNA fragments may be used. After purification, the material is measured and validated on standard assays: spectrophotometry (OD_{260nm}); gel electrophoresis; endotoxin quantitation by Limulus assay; bioactivity on human THP-1
25 cells (as in Example 28).

Example 31 shows that unexpectedly, different fractions of a selected synthetic RNA compound are endowed with different biological activity, based on size.

30 Differentiated human THP-1 monocytic cells were incubated with different concentrations of synthetic RNA (pA:pU, fractionated as described in the Example 30) for 24 hours, and the supernatants collected. The concentration of TNF-α was measured

by ELISA using BioSource International kits (Camarillo, CA). The results are expressed in Figure 32 as pg/ml (concentration) for each culture condition.

The results depicted in Fig. 32 show that lower molecular weight fractions of a selected synthetic RNA compound are endowed with higher biological activity, in terms of cytokine production, by human monocytic THP-1 cells.

Example 32. Selected synthetic RNA motifs have, unexpectedly, a different immune profile in regard to generation of anti-RNA antibodies.

BALB/c mice were immunized intraperitoneally and subcutaneously with 50µg + 50µg of hIgG and synthetic RNA (pI:pC or pA:pU) and serum samples were prepared 1 week later. As a control, mice injected with hIgG in saline were used. The anti-hIgG, and dsRNA IgG antibody titers against pA:pU, pI:pC, pA and hIgG were measured by ELISA. In brief, wells were coated with antigen (10µg/ml of hIgG or synthetic RNAs) and blocked with SeaBlock (Pierce, Rockford, IL, catalog # 37527). Serial dilutions of serum and bronchoalveolar lavage fluid were incubated for at least 2 hours at room temperature. After washing, the assay was developed with anti-mouse IgG antibody coupled with alkaline phosphatase (Sigma, cat# A7434) followed by addition of substrate (pNPP, Sigma, cat# N2765) and measurement by using an automatic microtiter plate reader (Molecular Devices, ThermoMax) equipped with SoftMax software.

The results are expressed in Figure 33 as mean \pm SEM of endpoint titers (n=3 / group). The results in Fig. 33 show that pI:pC but not pA:pU induced antibody response against itself, with a cross-reactive component against another RNA motif.

Example 33. *In vivo* loading of APC by recombinant IgG results in generation of Tc1 type of MHC class I responses only when additional conditions are satisfied.

BALB/c mice were immunized with 50ug of recIgG-NP(Kd) subcutaneously, admixed with 50ug of selected synthetic RNA (pA:pU or pI:pC). As a control, naive mice or mice immunized with recombinant IgG only were used. At 3 weeks after immunization, the T cell response was measured by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-

cytokine Abs (4ug/ml for anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with NP 147-155 peptide or just with media, to assess the background. Plates were incubated 72 hours at 37⁰ C, 5% CO2. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C.

The next day, the plates were washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The frequency of cytokine producing T cells reacting to NP peptide was measured and expressed against the amount of peptide used for stimulation. The results are expressed as means + SEM of triplicates (n=3 mice / group).

As shown previously in Figure 19, the administration of recombinant IgG bearing the NP MHC class I-restricted epitope resulted in generation of Tc2 immunity but not Tc1 response, implying *in vivo* formation of class I-peptide complexes with a specific co-stimulation profile. The results in Figures 34A and 34B show that co-use of selected synthetic RNAs promoted effective induction of IL-2 and IFN-gamma subsequent to IgG mediated delivery of an MHC class I-restricted epitope (dsRNA1 is pA:pU and dsRNA2 is pI:pC).

Example 34: Effective formation of MHC class I-peptides and instruction of the resulting T cell response by simultaneous manipulation of APC loading via Fcgamma R and activation via RNA receptors.

Splenic APC were isolated from naive BALBc mice and pulsed *ex vivo* overnight with 1 μ g NP peptide, or 50 μ g recIgG-NP (Kd) with or without 50 μ g/ml selected synthetic dsRNA (pA: pU). The cells were washed and 5×10^6 cells were administered by s.c. and i.p. injection equal amount, to naive BALB/c mice. The response was measured 3 weeks later by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4 μ g/ml for anti-IL4, and 8 μ g/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μ l/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200 μ l/well of DMEM complete containing FBS, for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5×10^5 /well together with 30 μ g/ml, 10 μ g /ml, or 3 μ g /ml NP peptide or just with media, to assess the background. Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 μ l /well of biotinylated anti-cytokine Abs, 2 μ g /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C. The next day the plates were washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results are expressed in Figure 35 as frequency of cytokine producing spot forming colonies against the concentration of peptide used for *ex vivo* stimulation (mean \pm SEM, n=3 mice /group). In addition, the mean area /colony versus the concentration of peptide used for stimulation is plotted, for both IFN-gamma and IL-4 (arbitrary units).

The results in Fig 35 show that *ex vivo* APC loading by recombinant IgG is significantly more effective in formation of MHC class I-peptide complexes and generation of Tc response, compared to use of peptide itself. In addition, the mere formation of MHC class I-peptide complexes subsequent to epitope delivery via IgG / FcgammaR results in differentiation of Tc2 cells producing IL-4 but not IFN-gamma.

Simultaneous treatment of APC with selected synthetic RNA results in broadening of the T cell profile, to IFN-gamma producing Tc1 cells.

Example 35 shows that co-priming with IgG-peptide together with a selected co-stimulatory motif resulted in more effective secondary expansion of MHC class I-restricted T cells subsequent of virus infection.

BALB/c mice were injected with recIgG-NP(Kd), pA:pU separately, or in combination (50 ug / injection). As a control, naive mice were used. Three weeks after treatment, the mice were infected with 104 TCID₅₀ of A/WSN/32 H1N1 influenza virus, via the respiratory tract. Four days after infection, the T cell profile in the spleen was measured by ELISPOT analysis subsequent to *ex vivo* stimulation with NP peptide as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4° C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37° C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 20 µg /ml NP peptide or just with media, to assess the background.

Plates were incubated 72 hours at 37° C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4° C. The next day the plates were washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results are expressed in Figure 36 as frequency of NP-specific MHC class I-restricted T cells forming cytokine producing colonies (means ± SEM, n=4 mice / group).

The results in Fig. 36 show that IgG mediated delivery of a class I restricted epitope is most effective in priming class I restricted Tc1 responses when co-administration of selected synthetic RNA was carried out. Such primed precursors were rapidly expanded subsequent to infection with influenza virus.

5

Example 36 shows that the most effective priming of cytotoxic lymphocytes recognizing an MHC class I-restricted epitope occurs by co-administration of selected RNA motif together with peptide epitope inserted within the IgG backbone.

10

BALBc mice were immunized and challenged with recIgG-NP (Kd) as in the previous Example and sacrificed 4 days after influenza virus infection. The splenocytes were prepared, suspended in HL-1 medium at 5 million / ml and co-incubated for 5 days with 10µg/ml of NP 147-155 peptide and in presence of 5U/ml of recombinant IL-2. Splenocytes from 4 mice / group were pooled and incubated in flasks.

15

After expansion, viable cells were recovered by Ficoll gradient centrifugation, washed and incubated for 5 hours in V-bottom plates, in various numbers, with a fixed number of sp20 target cells with or without NP peptide (20µg/ml). The supernatants were harvested after plate centrifugation, and the concentration of LDH measured by using a Promega kit (cat # G1780). The results are expressed as percent specific lysis at different E: T ratios (Effector to Target ratio).

20

The results in Fig. 37 show that effective priming of anti-viral cytotoxic T cells requires both effective *in vivo* loading of APC with class I restricted epitope delivered via IgG, together with appropriate instruction by selected synthetic RNA motif, namely pA:pU.

25

Example 37 shows that vaccination with an IgG bearing a viral MHC class I-restricted epitope, together with selected synthetic RNA motif, provided protection against infectious challenge with a prototype virus.

30

BALB/c mice were immunized with 50ug of recIgG-NP (Kd) together with 50ug of selected synthetic RNA (pA: pU), by subcutaneous injection. Three weeks after immunization, the mice were challenged with 10⁴ TCID₅₀ of infectious WSN influenza virus and sacrificed 5 days later. The pulmonary virus was titrated in lung homogenates

by standard MDCK hemagglutination assay as follows: on day one MDCK cells were plated in 96 well plates at 2×10^4 / well/ 200ul and incubated for 24 hours at 37° C, 5% CO_2 . The next day, 25 μl of the 10 fold dilutions in DMEM media of the lung homogenates were incubated in briefly trypsinized MDCK plates (1 minute) in triplicates and incubated at 37° C. After one hour, 175 μl of the DMEM complete media was added and plates were incubated for 48 hours at 37° C, 5% CO_2 . After two days, the hemagglutination-inhibition was done with chicken red blood cells incubated with the cell culture supernatants from the MDCK plate for 30 minutes at room temperature and the results were expressed as means \pm SEM of total pulmonary virus (n=4 mice / group). As a control, non-immunized mice were used.

The results in Fig. 38 show that immunization with a recombinant IgG bearing a viral class I restricted epitope together with selected synthetic dsRNA (pA:pU) resulted in priming of an immune response capable to limit the replication of a virus subsequent to infectious challenge.

Example 38. Figure 39 describes the tumor models used for testing the efficiency of a Ig-peptide-based molecules.

Balb-c mice (K^d restricted) have been used to establish a tumor model. Tumor cells (1 to 15 million in 100 μL) were typically injected in the flank to the mouse (see arrow in upper photo in Figure 39). Primary tumors (i.e. those at the sight of injection) were first detected by palpating the area and then quantitated by measuring the tumor size with a caliper (see Figure 39). In one series of experiments, the mouse myeloma cell line (SP2/0), either untransfected cells or cells stable transfected expressing heterologous protein (recombinant IgG expressing different epitope peptides in the CDR3 region of the heavy chain or the complete NP protein), was used to induce tumors in the mice. Expression of heterologous proteins in the SP2/0 cells provided specific tumor associated antigens (TAA) for testing various anti-tumor strategies in the immunocompetent mice. Typically, untreated mice developed palpable solid primary tumors 1 week post injection that led to morbidity and death over the next 4 weeks. Postmortem examination of the injected mice revealed metastatic lesions (see Figure 39). Sp2/0 cells were cultured from primary tumor tissue as well as spleen taken from tumor-bearing mice (data not shown).

SP2/0 cells were stably transfected with a recombinant IgG-expressing plasmids that were all identical except for the specific epitope sequence introduced into the CDR3 region of the heavy chain, for example, the MHC I restricted NP epitope (amino acids 147-155, see Figure 39). SP2/0 cells were also stably transfected with a plasmid
5 containing the coding sequence for the entire NP protein of WSN virus under control of the CMV promoter. All transfected cell lines produced primary tumors over the same frame as wild type SP2/0 cells.

This tumor model was extended to include an adenocarcinoma cell line (4T1, ATCC CRL-2539, K^d restricted), previously shown to induce metastatic tumors in Balb-c
10 mice. The 4T-1 cell line was similar to that described above for the SP/0 line. Injection of 1 to 15 million 4T-1 cells into the flank of Balb-c mice produced a palpable primary tumor over a time frame similar to injections of SP2/0 cells eventually leading to death. Postmortem collection of tissue from various organs showed that 4T-1 could be recovered from spleen, lungs as well as the primary tumor (not shown). 4T-1 cells were
15 stably transfected with a NP-expressing plasmid described above. As with SP2/0 cells, transfection of the 4T-1 cell did not affect the course of tumor growth and lethality of disease.

**Example 39 demonstrates successful control and treatment of a tumor after clinical
20 diagnosis, by using a tumor associate T cell epitope within a recombinant IgG together with a selected co-stimulatory RNA motif.**

Balb/c mice were injected with SP2/0 cells (15 million in 100 μ L) stably expressing recombinant IgG carrying the MHC I (Kd) NP epitope peptide in the CDR3
25 region of the heavy chain (IgNP). At day 7 post injection all mice had palpable tumors and the mice were randomized into 3 groups: co-stimulatory motif (i.e. dsRNA comprised of polymeric pApU) alone; purified IgTAA protein (IgNP); and both dsRNA pA:pU and purified IgTAA protein. The time of treatment is indicated by the arrows in Figure 40, and each injection contained 50 μ g of the indicated compound. The mice that
30 developed metastatic disease and died are represented with a "D" in the figure.

The data show that the combination of dsRNA (co-stimulatory motif) and IgTAA (IgNP) produced a dramatic protective response in mice that all had primary tumors at the

start of therapy. While all mice treated with either the dsRNA or IgTAA compound alone succumbed to disease, 100% of the mice treated with both were still alive 3 weeks after initiation of treatment and were in good clinical condition at the time of sacrifice for measurement of T cell response. These data show that *in vivo* loading of APC with TAA
5 (accomplished by uptake of IgNP via the Fc receptor of APC) is not sufficient for a potent anti-tumor response. The tumor rejection and survival displayed by mice treated with IgNP in combination with pApU dsRNA highlights the important role co-stimulation plays in treatment of tumors with tumor-associated antigens.

In conclusion, the results in Figure 40 show that both effective *in vivo* loading of
10 APC with tumor associated antigen, together with simultaneous activation by selected synthetic RNA motifs, are necessary and sufficient for effective control of tumor growth and induction of tumor rejection.

Example 40. This Example, in context of sublethal inoculation of tumor cells, shows that the suboptimal response to tumor antigens could be corrected by therapy with peptide epitope within an IgG backbone, together with co-stimulatory motif.
15

Balb/c mice were injected with SP2/0 cells stably expressing recombinant IgG (IgNP) that contains the MHC I (K^d) epitope (amino acids 147-155) of WSN virus
20 nucleoprotein in the CDR3 of the heavy chain. The cell inoculum was 1 million cells (in 100 μ L) per mouse. The mice were observed until such time as palpable tumors were detected at the site of injection. At this point the tumors were measured and 8 mice were left untreated (control) while 6 were injected intratumorally with purified IgTAA (i.e. purified IgNP, 2 mg/kg) and dsRNA (pApU, 4 mg/kg) weekly. Weekly measurements of
25 the tumors were taken.

Panel A of Figure 41 shows that in 6 of 8 of the control mice the induced tumor was progressive and ultimately lethal whereas 2 of the mice completely rejected the tumor spontaneously. Panel B of Figure 41 shows that the 3 weekly treatments with IgNP/dsRNA (indicated by the arrows) stimulated complete tumor rejection in 4 of the 6
30 mice and significant remission in another.

The results in Figure 41 shows that both effective *in vivo* loading of APC with tumor associated antigen, together with simultaneous activation by selected synthetic RNA, can trigger an effective immune response to tumor-associated antigens.

- 5 **Example 41 shows that therapy of tumor-bearing mice with a tumor epitope within an IgG backbone together with co-stimulatory synthetic dsRNA results in the restoration of the activatory status of tumor infiltrating lymphocytes.**

10 Two BALB/c mice were injected with 10 million sp20 transfectoma expressing the NP-K^d epitope. After tumors developed, one mouse was injected intratumorally with 50 µg of selected dsRNA motif (pApU) plus 50µg of "IgNP" - recIgG-NP(K^d) in saline. The mice were sacrificed 24 hours later, tumors excised, digested with collagenase, filtered through 70um filter and viable cells isolated on Ficoll gradient. Cells were stained with mAbs against TCRβ, CD25 or isotype control and assessed by FACS analysis. The
15 results were expressed as histograms, with percentage stained cells indicated.

Materials:

1. SP20 cell line (ATCC);
2. BALB/c mice (Harland Sprague Dawley);
- 20 2. Falcon 70 micron filter(Becton Dickinson, cat# 352350);
3. Collagenase (Sigma, cat# C-9891);
4. BSA, fraction V (Sigma, cat# A-4503);
5. Collagenase buffer: 0.225gm BSA + 0.00625gm in 50 ml RPMI;
6. Ficoll-hypaque (1.077, Amersham, cat# 17-1440-02);
- 25 7. FACS Buffer: 1% fetal calf serum + 0.1% azide in PBS;
8. Antibodies: All from BD Pharmingen; and,
9. Flow Cytometer: FACSCalibur (Becton Dickinson).

Method: Tumor cell isolation and FACS analysis:

- 30 1. Tumor was induced as stated above 6 weeks prior;
2. Tumor was isolated from BALB/c mouse;
3. Tumor was minced with sterile scissors and 10ml of collagenase buffer added;

4. Incubate 40 minutes, 37°C;
5. Force tumor through a 70µm Falcon filter with a 3ml syringe plunger into a 50ml tube while washing with RPMI;
6. Wash 1X and resuspend in 4 mls warm RPMI buffer;
- 5 7. With equal volume of cell suspension layered over Ficoll, centrifuge at RT, 2000 RPM, for 15 minutes;
8. Isolate layer and wash once in HL-1 buffer and resuspend in FACS buffer to 2×10^6 /ml and run flow cytometry analysis;
9. Remaining cells were used for ELISPOT analysis;
- 10 10. Cells were placed in 12X75mm tubes, 50µl/tube and stained with FITC labeled anti-mouse antibody, 2µg/ tube plus 1µl/tube mouse serum:
 - Isotypic Control;
 - Anti -CD40;
 - Anti -CD8;
 - 15 • Anti -CD4;
 - Anti -CD25;
 - Anti -TCR gamma delta;
 - Anti -TCR Beta;
11. Incubate 30 minutes on ice; and,
- 20 12. Wash once with FACS buffer and resuspend in 300µl FACS buffer.

The results in Figure 42 show that tumor infiltrating lymphocytes displaying the T cell receptor marker TCRβ acquired expression of the activation marker CD25 upon treatment with recombinant immunoglobulin bearing tumor associated epitope, together
25 with selected synthetic dsRNA motif.

Example 42 shows that successful therapy of tumor bearing mice with a peptide epitope within the IgG backbone together with a selected co-stimulatory molecule is associated with a specific differentiation pattern of Tc, comprising Tc1 in addition to Tc2.
30

Mice that successfully rejected the tumor following treatment with recombinant Ig carrying a tumor associated epitope together with selected synthetic dsRNA motif as explained in Example 40, were sacrificed and the T cell response against tumor associated epitope measured by ELISPOT analysis. The ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C.

Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with various concentrations of NP peptide. Plates were incubated 72 hours at 37. ⁰ C, 5% CO₂. After 3 days, plates were washed 5 times with PBS-tween20 0.05% (washing buffer), and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4 ⁰ C. The next day the plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. Plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results were expressed as number (mean ± SEM) of spot forming colonies corresponding to IL-4, IL-2 and IFN-γ. As a control, non-treated mice were used, which failed to reject tumor (n=4/group).

The results in Fig. 43 show that the treated mice that successfully rejected the tumor, developed Tc1 responses against the tumor associated epitope on the therapeutic Ig, along with Tc2 immunity. In contrast, the mice that failed to reject the tumor developed only Tc2 immunity.

Example 43 shows induction of effective memory response subsequent to specific treatment of tumor bearing mice with a T cell epitope within the IgG backbone, together with a selected co-stimulatory motif.

5 Mice bearing sp2/0 tumors expressing the NP-K^d TAA were treated as described in the Example 40, by injection with recombinant Ig bearing TAA together with selected synthetic RNA motifs. After tumor rejection, the mice were challenged by subcutaneous injection administered contralaterally, with 15 million SP2/0 cells expressing NP-Kd
10 epitope. In parallel, 4 control naïve mice were similarly injected with a tumorigenic / lethal dose of same type of cells. The development and size of the tumors was monitored and represented as diameter (mm) versus time since challenge.

The results in Figure 44 show that successful rejection of the tumor induced by indicated treatment is followed by effective protection against subsequent challenge with
15 the same tumor, indicating development of effective immune memory.

**Example 44 shows that surprisingly, the induction of tumor rejection by an IgG bearing a TAA together with a costimulator dsRNA motif, results in cross-protection against a range of tumor cell variants lacking the TAA or displaying
20 variants of TAA.**

The mice protected against homologous challenge as described in Example 43, were subjected to sequential challenge with 15 million tumor cells representing the same tumor cells devoid of TAA (loss of antigen mutants) or bearing variants of TAA lacking
25 the NP-K^d epitope. In addition, mice were challenged with a different type of tumor cell line (4T-1 adenocarcinoma) as a control, displayed in the table attached to Fig. 45A. In every case, naïve controls were included.

The status of T cell immunity of mice protected against multiple challenges with tumor variants, has been assessed by ELISPOT analysis using splenic cell suspensions
30 stimulated with TAA (NP-Kd peptide), HA (MHC class II-restricted peptide), or protein extracts from cell lysates. The ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight.

The next day, the plates were washed 2 times with DMEM media and blocked with 200 μ l/well of DMEM complete containing FBS, for an hour at 37⁰ C.

Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with various concentrations of antigen. Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 μ l /well of biotinylated anti-cytokine Abs, 2 μ g /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C. The next day the plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results were expressed as number (mean \pm SEM) of spot forming colonies corresponding to IL-4, IL-2 and IFN- γ . As a control, non-treated mice that failed to reject tumor (n=4/group) were used. As a control, naïve mice were included. The data are expressed as number (mean \pm SEM) of cytokine producing cells / organ (n=3/group).

The results in Fig. 45A - 45B (including the table in Fig. 45 A) show that the emerging immunity, subsequent to the indicated treatment that results in tumor rejection, protects against challenge with loss of antigen variants and is associated with overall expansion of cytokine producing cells. This indicates a broadening of the repertoire of anti-tumor lymphocytes, promoted by the proposed regimen, to tumor associated antigens that are not borne by the immunotherapeutic molecule.